PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 May 2001 (02.05.01)	
International application No. PCT/US00/18935	**Applicant's or agent's file reference** 2115S1131PCH
International filing date (day/month/year) 07 July 2000 (07.07.00)	**Priority date** (day/month/year) 09 July 1999 (09.07.99)
Applicant SHAYMAN, James, A. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

30 January 2001 (30.01.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer S. Mafla
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 211501131PCH	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/18935	International filing date *(day/month/year)* 07 July 2000 (07.07.2000)	Priority date *(day/month/year)* 12 July 1999 (12.07.1999)
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 31/40; 31/4025; A61P 35/04; C07D 295/12, 319/18 and US Cl.: 514/422, 428; 548/526, 568		
Applicant REGENTS OF THE UNIVERSITY OF MICHIGAN, THE TECHNOLOGY MANAGEMENT OFFICE		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30 January 2001 (30.01.2001)	20 July 2001 (20.07.2001)
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Richard L. Raymond Telephone No. (703) 308-1235

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International Application No.
PCT/US00/18935

I. Basis of the report

1. With regard to the elements of the international application:*

☒ the international application as originally filed.

☒ the description:
pages 1-44 as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the claims:
pages 45-47, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the drawings:
pages 1-12, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/18935

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-17	YES
	Claims NONE	NO
Inventive Step (IS)	Claims 1-17	YES
	Claims NONE	NO
Industrial Applicability (IA)	Claims 1-17	YES
	Claims NONE	NO

2. CITATIONS AND EXPLANATIONS (Rule 70.7)

Claims 1-17 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the present specific compounds, D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and D-*t*-4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, and their uses in inhibiting the growth of cancer cells, treating sphingolipidosis, treating infections and use in a vaccination method.

The demand must be filed directly with with the one chosen by the applicant.

competent International Preliminary Examining Authority or two or more Authorities are competent, name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EPO

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: 211501131POH

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/US00/18935	07 July 2000 (07/07/2000)	09 July 1999 (09/07/1999)

Title of invention:
AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

The Regents of the University of Michigan
Technology Management Office
2071 Wolverine Tower
3003 S. State Street
Ann Arbor, MI 48109-1280
US

Telephone No.: 734-647-5234
Facsimile No.: 734-936-1330
Teleprinter No.:

State *(that is, country)* of nationality: US
State *(that is, country)* of residence: US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SHAYMAN, James A.
1890 Snowberry Ridge Road
Ann Arbor, MI 48103
US

State *(that is, country)* of nationality: US
State *(that is, country)* of residence: US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

RADIN, Norman S.
350 Sharon Park Dr.
Apt. S5
Menlo Park, CA 94025-6802

State *(that is, country)* of nationality: US
State *(that is, country)* of residence: US

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/US00/18935

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [x] agent [ ] common representative

and [x] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GIBBS, Barbara S.
Harness, Dickey & Pierce, P.L.C.
P. O. Box 828
Bloomfield Hills, MI 48303
US

Telephone No.: 248-641-1600

Facsimile No.: 248-641-0270

Teleprinter No.: 287637 HARNES UR

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special addr ess to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

[x] the international application as originally filed

the description [x] as originally filed
[ ] as amended under Article 34

the claims [x] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [x] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

[x] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

Sheet No. 3.

International application No.
PCT/US00/18935

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*: Postcard, Exp. Mail Certificate

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Barbara S Gibbs

BARBARA S. GIBBS, Ph.D., Reg. No. 44,708

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. [ ] The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. [ ] The applicant has been informed accordingly.
4. [ ] The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. [ ] Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No.	PCT/US00/18935
Applicant's or agent's file reference	211501131POH

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant

THE REGENTS OF THE UNIVERSITY OF MICHIGAN;
SHAYMAN, James A. and RADIN, Norman S.

Calculation of prescribed fees

1. Preliminary examination fee	750.00	P
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	137.00	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	887.00	TOTAL

Mode of Payment

☐	authorization to charge deposit account with the IPEA (see below)	☐	cash
☒	cheque	☐	revenue stamps
☐	postal money order	☐	coupons
☐	bank draft	☐	other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ EPO ☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

08-0750	30 January, 2001	Barbara Smith
Deposit Account Number	Date *(day/month/year)*	Signature

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
2115S1131PCH		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/ 18935	07/07/2000	09/07/1999
Applicant		
REGENTS OF THE UNIVERSITY OF MICHIGAN...		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

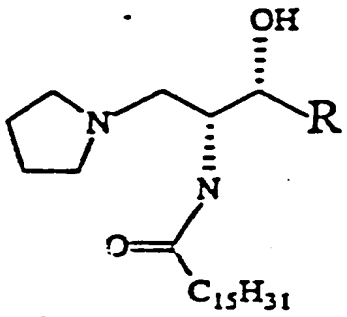
   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

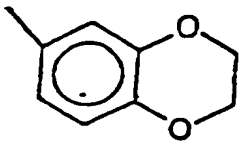
International application No.
PCT/US 00/18935

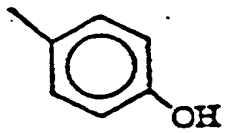
Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

Novel amino ceramide-like compounds :



R=





are provided which inhibit glucosyl ceramide (GlcCer) formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of glycosphingolipids. The compounds of the present invention have improved GlcCer synthase inhibition activity and are therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered glycosphingolipid levels.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/04108 A1

(51) International Patent Classification[7]: C07D 319/18, 295/12, A61K 31/40, 31/4025, A61P 35/04

(21) International Application Number: PCT/US00/18935

(22) International Filing Date: 7 July 2000 (07.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/350,678 9 July 1999 (09.07.1999) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/350,678 (CON)
Filed on 9 July 1999 (09.07.1999)

(71) Applicant *(for all designated States except US)*: **REGENTS OF THE UNIVERSITY OF MICHIGAN** [US/US]; Technology Management Office, Room 2071, Wolverine Tower, 3003 South State Street, Ann Arbor, MI 48109-1280 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHAYMAN, James, A.** [US/US]; 1890 Snowberry Ridge Road, Ann Arbor, MI 48103 (US). **RADIN, Norman, S.** [US/US]; 350 Sharon Park Drive, Apt. S5, Menlo Park, CA 94025-6802 (US).

(74) Agents: **GIBBS, Barbara, S.** et al.; Harness, Dickey & Pierce, P.L.C., P.O. Box 828, Bloomfield Hills, MI 48303 (US).

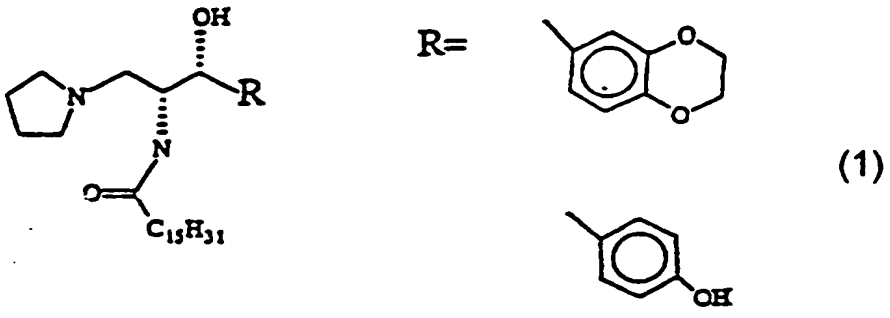
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE



(1)

(57) Abstract: Novel amino ceramide-like compounds (1) are provided which inhibit glucosyl ceramide (GlcCer) formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of glycosphingolipids. The compounds of the present invention have improved GlcCer synthase inhibition activity and are therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered glycosphingolipid levels.

WO 01/04108 A1

AMINO CERAMIDE - LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE

RELATED APPLICATIONS

The present application is a continuation-in-part of U.S. Serial No. 08/883,218, filed June 26, 1997, which is a divisional of U.S. Serial No. 08/708,574, filed September 5, 1996, now U.S. Patent No. 5,916,911, which claims priority from U.S. Serial No. 60/004,047, filed September 20, 1995, all of which are hereby expressly incorporated by reference.

SPONSORSHIP

The present invention was supported by grant nos. R01 DK41487, R01 DK69255 and RO139255 from the National Institutes of Health, contract R43 CA 58159 from the National Cancer Institute, grant GM 35712 from the National Institute of General Medical Sciences, and by the University of Michigan Comprehensive Cancer Center grant 2P30 CA 46592 from the National Cancer Institute, U.S. Public Health Service, DHHS. Grant number for Merit Award from Veteran's Administration? The govemment may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to ceramide-like compounds and, more particularly, to ceramide-like compounds containing a tertiary amine group and their use in therapeutic methods.

BACKGROUND OF THE INVENTION

Hundreds of glycosphingolipids (GSLs) are derived from glucosylceramide (GlcCer), which is enzymatically formed from ceramide and UDP-glucose. Th enzyme involved in GlcCer formation is UDP-glucose:N-acylsphingosine glucosyltransferase (GlcCer synthase). The rate of GlcCer formation under physiological conditions may depend on the tissue level of UDP-glucose, which in tum depends on the level of glucose in a particular tissue (Zador, I.Z. et al., "A Role for Glycosphingolipid Accumulation in the Renal Hypertrophy of Streptozotocin-Induced Diabetes Mellitus," *J. Clin. Invest.* 91:797-803 (1993)). *In vitro* assays based on endogenous ceramide yield lower synthetic rates than mixtures containing added ceramide, suggesting that tissue levels of ceramide are also normally rate-limiting (Brenkert, A. et al., "Synthesis of Galactosyl Ceramide and Glucosyl Ceramid by Rat Brain: Assay Procedures and Changes with Age," *Brain Res.* 36:183-193 (1972)).

It has been found that the level of GSLs controls a variety of cell functions, such as growth, differentiation, adhesion between cells or betwe n cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor

cells. In addition, the GlcCer precursor, ceramide, may cause differentiation or inhibition of cell growth (Bi lawska, A. t al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters* 307:211-214 (1992)) and be involved in the functioning of vitamin D_3, tumor necrosis factor-*α*, interleukins, and apoptosis (programmed cell death). The sphingols (sphingoid bases), precursors of ceramide, and products of ceramide catabolism, have also been shown to influence many cell systems, possibly by inhibiting protein kinase C (PKC).

It is likely that all the GSLs undergo catabolic hydrolysis, so any blockage in the GlcCer synthase should ultimately lead to depletion of the GSLs and profound changes in the functioning of a cell or organism. An inhibitor of GlcCer synthase, PDMP (1R-phenyl-2R-decanoylamino-3-morpholino-1-propanol), previouslyidentified as the D-*threo* isomer (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987)), has been found to produce a variety of chemical and physiological changes in cells and animals (Radin, N.S. et al., "Use of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol (PDMP), an Inhibitor of Glucosylceramide Synthesis," *In NeuroProtocols, A Companion to Methods in Neurosciences*, S. K. Fisher et al., Ed., (Academic Press, San Diego) 3:145-155 (1993) and Radin, N.S. et al., "Metabolic Effects of Inhibiting Glucosylceramide Synthesis with PDMP and Other Substances," *In Advances in Lipid Research; Sphingolipids in Signaling, Part B.*, R.M. Bell et al., Ed. (Academic Press, San Diego) 28:183-213 (1993)). Particularly interesting is the compound's ability to cure mice of cancer induced by Ehrlich ascites carcinoma cells (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.* 38:23-30 (1987)), to produce accumulation of sphingosine and *N,N*-dimethylsphingosine (Felding-Habermann, B. et al., "A Ceramide Analog Inhibits T Cell Proliferative Response Through Inhibition of Glycosphingolipid Synthesis and Enhancement of *N,N*-Dimethylsphingosine Synthesis," *Biochemistry* 29:6314-6322 (1990)), and to slow cell growth (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). Compounds with long r chain fatty acyl groups have been found to be substantially more effective (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synth sis," *J. Biochem.* 111:191-196 (1992)).

The importance of GSL metabolism is underscored by the seriousness of disorders resulting from defects in GSL metabolizing enzymes. For example, Tay-Sachs, Gaucher's, and Fabry's diseases, resulting from enzymatic defects in the GSL degradative pathway and the accumulation of GSL in the patient, all have severe clinical manifestations. Another example of the importance of GSL function is seen in a mechanism by which blood cells, whose surfaces contain selectins, can, under certain conditions, bind to GSLs in the blood vessel walls and produce acute, life-threatening inflammation (Alon, R. et al., "Glycolipid Ligands for Selectins Support Leukocyte Tethering & Rolling Under Physiologic Flow Conditions." *J. Immunol.*, 154:5356-5366 (1995)).

At present there is only one treatment available for patients with Gaucher disease, wherein the normal enzyme which has been isolated from normal human tissues or cultured cells is administered to the patient. As with any drug isolated from human material, great care is needed to prevent contamination with a virus or other dangerous substances. Treatment for an individual patient is extremely expensive, costing hundreds of thousands, or even millions of dollars, over a patient's lifetime. It would thus be desirable to provide a treatment which includes administration of a compound that is readily available and/or producible from common materials by simple reactions.

Possibly of even greater clinical relevance is the role of glucolipids in cancer. For example, it has been found that certain GSLs occur only in tumors; certain GSLs occur at abnormally high concentrations in tumors; certain GSLs, added to tumor cells in culture media, exert marked stimulatory or inhibitory actions on tumor growth; antibodies to certain GSLs inhibit the growth of tumors; the GSLs that are shed by tumors into the surrounding extracellular fluid inhibit the body's normal immunodefense system; the composition of a tumor's GSLs changes as the tumors become increasingly malignant; and, in certain kinds of cancer, the level of a GSL circulating in the blood gives useful information regarding the patient's response to treatment. Because of the significant impact GSLs have on several biochemical processes, there remains a need for compounds having improved GlcCer synthase inhibition activity.

It would thus be desirable to provide compounds which inhibit GlcCer synthas activity. It would also be desirable to provid compounds which inhibit GlcCer synthase activity, thereby lowering the level of GSLs and increasing GSL precursor levels, *e.g.* increasing the levels of ceramide and sphingols. It would further b

desirable to provide compounds which inhibit GlcCer synthase activity and lower the level of GSLs without also increasing ceramide levels. It would also be desirable to provide compounds and therapeutic methods to treat conditions and diseases associated with altered GSL levels and/or GSL precursor levels.

SUMMARY OF THE INVENTION

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibition activity and are therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels, as well as GSL precursor levels. For example, the compounds of the present invention may be useful in methods involving cancer growth and metastasis, the growth of normal tissues, the ability of pathogenic microorganisms to bind to normal cells, the binding between similar cells, the binding of toxins to human cells, and the ability of cancer cells to block the normal process of immunological cytotoxic attack.

Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

Figure 1 is a graph showing the growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors;

Figure 2 is a graph showing the protein content of MDCK cells cultured for 24 hr in medium containing different concentrations of the separated *erythro*- and *threo*-isomers of a preferred compound of the present invention;

Figure 3 is a graph showing [^{3}H]thymidine incorporation into the DNA of MDCK cells treated with a preferred compound of the present invention;

Figures 4A and 4B are graphs showing the effects of P4 and *p*-methoxy-P4 on GlcCer synthase activity;

Figure 5 is a graph showing the linear relationship between the inhibition of GlcCer synthase activity and electronic parameter (δ) and hydrophobic parameter (π);

Figure 6 is a graph showing the effects of dioxy P4 derivatives on GlcCer synthase activity;

Figure 7 is a bar graph showing the effects of D-*t*-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth;

Figure 8 is a schematic of the synthetic pathway for 4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol;

Figure 9 is an illustration of the structures of P4 and of phenyl-substituted P4 homologues;

Figure 10 is an HPLC chromatogram showing the separation of the enantiomers of P4 and p-methoxy-P4 by chiral chromatography;

Figure 11 is a graph showing the effects of D-*threo*-4'-hydroxy-P4 as compared to D-*threo*-p-methoxy-P4 on GlcCer synthase activity;

Figure 12 is a graph showing the effects of D-*threo* enantiomers of P4, 4'-hydroxy-P4 and 3',4'-ethylenedioxy-P4 on 1-O-acyceramide synthase activity;

Figure 13 is a graph showing the effect of D-*threo*-P4 on GlcCer synthesis and cell growth;

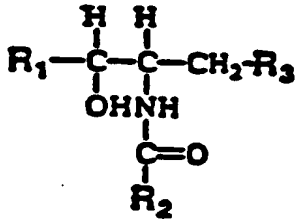
Figure 14 is a graph showing the effect of D-*threo*-4'-hydroxy-P4 on GlcCer synthesis and cell growth; and

Figure 15 is a graph showing the effect of D-*threo*-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibitory activity and are therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels.

The compounds of the present invention generally have the following formula:



wherein

R_1 is a phenyl group, preferably a substituted phenyl group such as p-methoxy, hydroxy, dioxane substitutions such as methylenedioxy, thylen dioxy, and trimethyl nedioxy, cyclohexyl or other acyclic group, t-butyl or other branched aliphatic

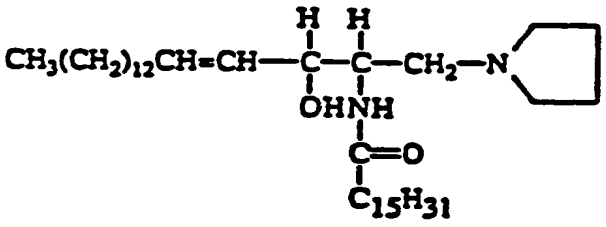
group, or a long alkyl or alkenyl chain, preferably 7 to 15 carbons long with a double bond next to the k mel of the structure. The aliphatic chain can have a hydroxyl group near the two asymmetric centers, corresponding to phytosphingosine.

R_2 is an alkyl residue of a fatty acid, 10 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (*e.g.*, a hydroxyl group).

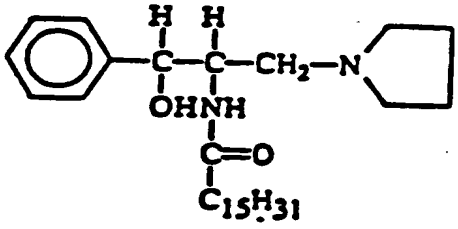
R_3 is a tertiary amine, preferably a cyclic amine such as pyrrolidine, azetidine, morpholine or piperidine, in which the nitrogen atom is attached to the kernel (*i.e.*, a tertiary amine).

All four structural isomers of the compounds are contemplated within the present invention and may be used either singly or in combination (*i.e.*, DL-*threo* or DL-*erythro*).

The preferred aliphatic compound of the present invention is D-*threo*-1-pyrrolidino-1-deoxyceramide, identified as IV-231B herein and also referred to as PD. The preferred aromatic compound of the present invention is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, identified as BML-119 herein and also referred to as P4. The structures of the preferred compounds are as follows:



PD



P4

An additional preferred compound of the present invention are D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, also referred to herein as D-*t*-3',4'-ethylenedioxy-P4, and D-t-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, also referred to herein as D-t-4'-hydroxy-P4.

By increasing the acyl chain length of PDMP from 10 to 16 carbon atoms, the efficacy of the compounds of the present invention as GlcCer synthase inhibitors is greatly enhanced. The use of a less polar cyclic amine, especially a pyrrolidine instead of a morpholine ring, also increases the efficacy of the compounds. In addition, replacement of the phenyl ring by a chain corresponding to sphingosine yields a strongly inhibitory material. By using a chiral synth tic route, it was discovered that the isomers active against GlcCer synthas had the

R,R-(D-*threo*)-configuration. However, strong inhibition of the growth of human cancer cells *in plastico* was produced by both the *threo* and *erythro* racemic compounds, showing involvement of an additional factor beyond simple depletion of cell glycosphingolipids by blockage of GlcCer synthesis. The growth arresting effects could be correlated with increases in cellular ceramide and diglyceride levels.

Surprisingly, the aliphatic pyrrolidino compound of the present invention (identified as IV-231B), was strongly inhibitory toward the GlcCer synthase and produced almost complete depletion of glycolipids, but did not inhibit growth or cause an accumulation of ceramide. Attempts were made to determine if the differences in growth effects could be attributed to the influence of the inhibitors on related enzymes (ceramide and sphingomyelin synthase and ceramidase and sphingomyelinase). While some stimulation or inhibition of enzyme activity was noted, particularly at high inhibitor concentrations (50 μM), these findings did not explain the differing effects of the different inhibitors.

By slowing the synthesis of GlcCer, the compounds of the present invention lower the levels of all the GlcCer-derived GSLs due to the GSL hydrolases which normally destroy them. While the body will continue to make the more complex GSLs from available GlcCer, the rate of synthesis will slow down as the level of GlcCer diminishes. The rate of lowering depends on the normal rate of destruction of each GSL. These rates however, are relatively rapid in animals and cultured cells.

At higher dosages, many of the compounds of the present invention produce an elevation in the level of ceramide. Presumably this occurs because cells continue to make ceramide despite their inability to utilize it for GlcCer synthesis. Ceramide is also normally converted to sphingomyelin, but this process does not seem to be able to handle the excess ceramide. It has been unexpectedly found however, that an additional process is also involved, since even those isomers that are inert against GlcCer synthase also produce an elevation in ceramide levels. Moreover, the blockage of GlcCer synthase can occur at low inhibitor dosages, yet ceramide accumulation is not produced. The preferred aliphatic compound of the present invention, D-*threo*-1-pyrrolidino-1-deoxyceramide (PD), does not produce ceramide accumulation at all, despite almost complete blockage of GlcCer synthesis.

This distinction between the aromatic and the aliphatic compounds of the present invention is important because ceramide has recently been proposed to cause cell death (apoptosis) by some still unknown mechanism. At lower dose levels, the aromatic compounds of the present invention cause GSL disappearance with only

small accumulation of ceramide and inhibition of cell growth. Higher dosages cause much more ceramide deposition and very slow cell growth or cell death.

In one embodiment of the present invention, methods of treating patients suffering from inborn genetic errors in the metabolism of GlcCer and its normal anabolic products (lactosylceramide and the more complex GSLs) are provided. The presently known disorders in this category include Gaucher, Fabry, Tay-Sachs, Sandhoff, and GM1 gangliosidosis. The genetic errors lie in the patient's inability to synthesize a hydrolytic enzyme having normal efficiency. Their inefficient hydrolase allows the GSL to gradually accumulate to a toxic degree, debilitating or killing the victim. The compounds of the present invention slow the formation of GSLs, thus allowing the defective hydrolase to gradually "catch up" and restore the concentrations of GSLs to their normal levels and thus the compounds may be administered to treat such patients.

With respect to Gaucher disease, it has been calculated that much of the patient's accumulated GlcCer in liver and spleen arises from the blood cells, which are ultimately destroyed in these organs after they have reached the end of their life span. The actual fraction, lipid derived from blood cells versus lipid formed in the liver and spleen cells, is actually quite uncertain, but the external source must be important. Therefore it is necessary for the compounds of the present invention to deplete the blood cells as they are formed or (in the case of white blood cells) while they still circulate in the blood. Judging from toxicity tests, the white cells continue to function adequately despite their loss of GSLs. Although the toxicity studies were not of a long enough duration to produce many new red cells with low GSL content, it is possible that circulating red cells also undergo turnover (continual loss plus replacement) of GSLs.

In an alternative embodiment of the present invention, for the treatment of disorders involving cell growth and division, high dosages of the compounds of the present invention are administered but only for a relatively short time. These disorders include cancer, collagen vascular diseases, atherosclerosis, and the renal hypertrophy of diabetic patients. Accumulation or changes in the cellular levels of GSLs have been implicated in these disorders and blocking GSL biosynthesis would allow the normal restorative mechanisms of the body to resolve the imbalance.

With atherosclerosis, it has been shown that arterial pithelial cells grow faster in the presence of a GlcCer product (lactosylceramide). Oxidized serum lipoprotein, a material that normally circulates in the blood, stimulates the formation of plaques

and lactosylceramide in the inner lining of blood vessels. Treatment with the compounds of the present invention would inhibit this mitogenic effect.

In an additional embodiment of the present invention, patients suffering from infections may be treated with the compounds of the present invention. Many types of pathogenic bacteria have to bind to specific GSLs before they can induce their toxic effects. As shown in Svensson, M. et al., "Epithelial Glucosphingolipid Expression as a Determinant of Bacterial Adherence and Cytokine Production," *Infect. and Immun.* 62:4404-4410 (1994), expressly incorporated by reference, PDMP treatment reduces the adherence of *E. coli* to mammalian cells. Several viruses, such as influenza type A, also must bind to a GSL. Several bacterial toxins, such as the verotoxins, cannot themselves act without first binding to a GSL. Thus, by lowering the level of GSLs, the degree of infection may be ameliorated. In addition, when a patient is already infected to a recognizable, diagnosable degree, the compounds of the present invention may slow the further development of the infection by eliminating the binding sites that remain free.

It has been shown that tumors produce substances, namely gangliosides, a family of GSLs, that prevent the host *i.e.*, patient, from generating antibodies against the tumor. By blocking the tumor's ability to secrete these substances, antibodies against the tumor can be produced. Thus, by administering the GlcCer synthase inhibitors of the present invention to the patient, the tumors will become depleted of their GSLs and the body's normal immunological defenses will come into action and destroy the tumor. This technique was described in Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.* 38:23-30(1987), expressly incorporated by reference. The compounds of the present invention and in particular the aliphatic compounds require much lower doses than those previously described. This is particularly important because the lower dose may reduce certain side effects. Moreover, because the aliphatic compounds of the present invention do not produce ceramide accumulation, they are less toxic. In addition, 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), may act via two pathways, GSL depletion and ceramide accumulation.

In an alternative embodiment, a vaccine-like preparation is provided. Here, cancer cells are removed from the patient (preferably as compl tely as possible), and th cells are grown in culture in order to obtain a large number of the cancer cells. The cells are then exposed to the inhibitor for a time sufficient to deplete the cells of their GLSs (generally 1 to 5 days) and are reinjected into the patient. These

reinjected cells act like antigens and are d stroyed by the patient's immunodefense system. The remaining cancer cells (which could not be physically removed) will also be attacked by the patient's antibodies. In a preferred embodiment, the patient's circulating gangliosides in the plasma are removed by plasmapheresis, since the circulating gangliosides would tend to block the immunodefense system.

It is believed that tumors are particularly dependent on GSL synthesis for maintenance of their growth (Hakomori, S. "New Directions in Cancer Therapy Based on Aberrant Expression of Glycosphingolipids: Anti-adhesion and Ortho-Signaling Therapy," *Cancer Cells* 3:461-470 (1991)). Accumulation of ceramide in treated tumors also slows their growth or kills them. Tumors also generate large amounts of GSLs and secrete them into the patient's body, thereby preventing the host's normal response by immunoprotective cells, which should generate antibodies against or otherwise destroy tumor cells (*e.g.*, tumors are weakly antigenic). It has also been shown that GSL depletion blocks the metastasis of tumor cells (Inokuchi, J. et al., "Inhibition of Experimental Metastasis of Murine Lewis Long Carcinoma by an Inhibitor of Glucosylceramide Synthase and its Possible Mechanism of Action," *Cancer Res.* 50:6731-6737 (1990). Tumor angiogenesis (*e.g.*, the production of blood capillaries) is strongly influenced by GSLs (Ziche, M. et al., "Angiogenesis Can Be Stimulated or Repressed in *In Vivo* by a Change in GM3:GD3 Ganglioside Ratio," *Lab. Invest.* 67:711-715 (1992)). Depleting the tumor of its GSLs should block the tumors from generating the new blood vessels they need for growth.

A further important characteristic of the compounds of the present invention is their unique ability to block the growth of multidrug resistant ("MDR") tumor cells even at much lower dosages. This was demonstrated with PDMP by Rosenwald, A.G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, on Lysosomes in Cultured Cells," *J. Lipid Res.* 35:1232 (1994), expressly incorporated by reference. Tumor cells that survive an initial series of therapeutic treatments often reappear some years later with new properties - they are now resistant to a second treatment schedule, even with different drugs. This change has been attributed to th appearance in the tumor of large amounts of a specific MDR protein (P-glycoprotein). It has been suggested that protein kinase C (PKC) may be involved in the action or formation of P-glycoprotein (Blobe, G.C. et al., "Regulation of PKC and Its Role in Cancer Biology," *Cancer Metastasis Rev.* 13:411-431 (1994)). However d creases in PKC have other important effects, particularly slowing of growth. It is known that PDMP does lower the cellular content of PKC (Shayman, J.A. t al., "Modulation f

Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)) but it is not clear why it so effectively blocks growth of MDR cells (Rosenwald, A.G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, On Lysosomes in Cultured Cells," *J. Lipid Res.* 35:1232 (1994)). A recent report showed that several lipoidal amines that block MDR action also lower the level of the enzyme acid sphingomyelinase (Jaffrezou,J. et al., "Inhibition of Lysosomal Acid Sphingomyelinase by Agents which Reverse Multidrug Resistance," *Biochim. Biophys. Acta* 1266:1-8 (1995)). One of these agents was also found to increase the cellular content of sphingosine 5-fold, an effect seen with PDMP as well. One agent, chlorpromazine, behaves like the compounds of the present invention, in its ability to lower tissue levels of GlcCer (Hospattankar, A.V. et al., "Changes in Liver Lipids After Administration of 2-Decanoylamino-3-Morpholinopropiophenone and Chlorpromazine," *Lipids* 17:538-543 (1982)).

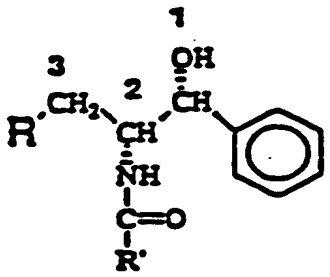
It will be appreciated by those skilled in the art that the compounds of the present invention can be employed in a wide variety of pharmaceutical forms; the compound can be employed neat or admixed with a pharmaceutically acceptable carrier or other excipients or additives. Generally speaking, the compound will be administered orally or intravenously. It will be appreciated that therapeutically acceptable salts of the compounds of the present invention may also be employed. The selection of dosage, rate/frequency and means of administration is well within the skill of the artisan and may be left to the judgment of the treating physician or attending veterinarian. The method of the present invention may be employed alone or in conjunction with other therapeutic regimens. It will also be appreciated that the compounds of the present invention are also useful as a research tool *e.g.*, to further investigate GSL metabolism.

The following Specific Example further describes the compounds and methods of the present invention.

SPECIFIC EXAMPLE 1

The following formulas set forth preferred aromatic and aliphatic compounds:

FORMULA I



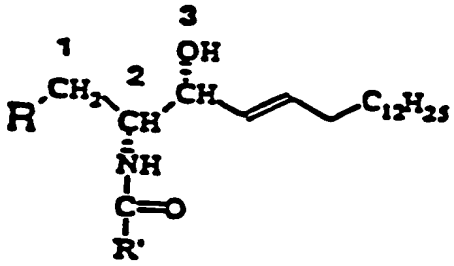
identifi d as (1R,2R)-1-phenyl-2-acylamino-3-cyclic amino-1-propanol, and referred to herein as the "aromatic inhibitors," wherein

The phenyl group can be a substituted phenyl group (such as *p*-methoxyphenyl).

R' is an alkyl residue of a fatty acid, 10 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (*e.g.*, a hydroxyl group).

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.

FORMULA II



identified as (2R,3R)-2-palmitoyl-sphingosyl amine or 1-cyclic amino-1-deoxyceramide or 1-cyclic amino-2-hexadecanoylamino-3-hydroxy-octadec-4,5-ene, and referred to herein as the "aliphatic inhibitors," wherein

R' is an alkyl residue of a fatty acid, 10 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (*e.g.*, a hydroxyl group).

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.

The long alkyl chain shown in Formula II can be 8 to 18 carbon atoms long, with or without a double bond near the asymmetric carbon atom (carbon 3). Hydroxyl groups can, with advantage, be substituted along the aliphatic chain, particularly on carbon 4 (as in the naturally occurring sphingol, phytosphingosine). The long chain can also be replaced by other aliphatic groups, such at t-butyl or cyclopentyl.

Th aromatic inhibitors (see Formula I and Table 1) were synthesized by the Mannich reaction from 2-*N*-acylaminoacetophenone, paraformaldehyde, and a secondary amine as previously described (Inokuchi, J. et al., "Preparation of the

Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetas ," *J. Lipid Res.* 28:565-571 (1987) and Vunnam, R.R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids* 26:265-278 (1980)). For those syntheses in which phenyl-substituted starting materials were used, the methyl group in the acetophenone structure was brominated and converted to the primary amine. Bromination of p-methoxyacetophenone was performed in methanol. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, MO. Miscellaneous reagents were from Sigma Chemical Co. and the sphingolipids used as substrates or standards were prepared by methods known in the art. The reactions produce a mixture of four isomers, due to the presence of two asymmetric centers.

The aliphatic inhibitors (See Formula II and Table 2) were synthesized from the corresponding 3-*t*-butyldimethylsilyl-protected sphingols, prepared by enantioselective aldol condensation (Evans, D.A. et al., "Stereoselective Aldol Condensations Via Boron Enolates," *J. Am. Chem. Soc.* 103:3099-3111 (1981) and Abdel-Magid, A. et al., Metal-Assisted Aldol Condensation of Chiral A-Halogenated Imide Enolates: A Stereocontrolled Chiral Epoxide Synthesis," *J. Am. Chem. Soc.* 108:4595-4602 (1986)) using a modification of the procedure of Nicolaou et al. (Nicolaou, K.C. et al., "A Practical and Enantioselective Synthesis of Glycosphingolipids and Related Compounds. Total Synthesis of Globotriaosylceramide (Gb_3)," *J. Am. Chem. Soc.* 110:7910-7912 (1988)). Each protected sphingol was first converted to the corresponding primary triflate ester, then reacted with a cyclic amine. Subsequent *N*-acylation and desilylation led to the final products in good overall yield (Carson, K.G. et al., "Studies on Morpholinosphingolipids: Potent Inhibitors of Glucosylceramide Synthase," *Tetrahedron Lett.* 35:2659-2662 (1994)). The compounds can be called 1-morpholino-(or pyrrolidino)-1-deoxyceramides.

Labeled ceramide, decanoyl sphingosine, was prepared by reaction of the acid chloride and sphingosine (Kopaczyk, K. C. et al., "*In Vivo* Conversions of Cerebroside and Ceramide in Rat Brain," *J. Lipid Res.* 6:140-145 (1965)) and NBD-SM (12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]- sphingosylphosphorylcholine) was from Molecular Probes, Inc., Eugene, OR.

Meth ds

TLC of the amines was carried out with HPTLC plates (E. Merck silica gel 60) and C-M-HOAc 90:10:10 (solvent A) or 85:15:10 (solvent B) or C-M-conc. ammonium

hydroxide 30:10:1 (solvent C). The bands were stained with iodine or with Coomassie Brilliant Blue R-250 (Nakamura, K. et al., "Coomassie Brilliant Blue Staining of Lipids on Thin-Layer Plates," *Anal. Biochem.* 142:406-41 (1984)) and, in the latter case, quantified with a Bio-Rad Model 620 videodensitometer operated with reflected white light. The faster band of each PDMP analog, previously identified as the *erythro* form, corresponds to the 1S,2R and 1R,2S stereoisomers, and the slower band, previously identified as the *threo* form, corresponds to the 1R,2R and 1S,2S stereoisomers.

TLC of the cell lipids was run with C-M-W 24:7:1 (solvent D) or 60:35:8 (solvent E).

Growth of cell lines. Comparisons of different inhibitors with regard to suppression of human cancer cell growth were made by the University of Michigan Cancer Center in vitro Drug Evaluation Core Laboratory. MCF-7 breast carcinoma cells, HT-29 colon adenocarcinoma cells, H-460 lung large cell carcinoma cells, and 9L brain gliosarcoma cells were grown in RPMI 1640 medium with 5% fetal bovine serum, 2 mM glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 0.1 mg/ml of neomycin. UMSCC-10A head and neck squamous carcinoma cells were grown in minimal essential medium with Earle salts and the same supplements. Medium components were from Sigma Chemical Co. Cells were plated in 96-well microtiter plates (1000 cells/well for H-460 and 9L cells, and 2000 cells/well for the other lines), and the test compounds were added 1 day later. The stock inhibitor solutions, 2 mM in 2 mM BSA, were diluted with different amounts of additional 2 mM BSA, then each solution was diluted 500-fold with growth medium to obtain the final concentrations indicated in the Figures and Tables.

Five days after plating the H-460 and 9L cells, or 6 days for the other lines, cell growth was evaluated by staining the adhering cells with sulforhodamine B and measuring the absorbance at 520 nm (Skehan, P. et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," *J. Natl. Cancer Inst.* 82:1107-1112 (1990)). The absorbance of the treated cultures is reported as percent of that of control cultures, to provide an estimate of the fraction of the cells that survived, or of inhibition of growth rate.

For the experiments with labeled thymidine, each 8.5 cm dish contained 500,000 Madin-Darby canine kidney (MDCK) cells in 8 ml of Dulb cco modified essential supplemented medium. The cells were incubated at 37°C in 5% CO_2 for 24 h, then incubated another 24 h with medium containing the inhibitor-BSA complex. The control cells were also incubated in the presence of BSA. Th cells were washed

with phosphate/saline and trichloroacetic acid, then scrap d off the dishes, dissolved in alkali, and analyzed for protein and DNA incorporated tritium. [Methyl-^{3}H]thymidin (10 μCi) was added 4 h prior to harvesting.

Assay of sphingolipid enzymes. The inhibitors were evaluated for their effectiveness against the GlcCer synthase of MDCK cell homogenates by incubation in a thermostatted ultrasonic bath (Radin N.S. et al., "Ultrasonic Baths as Substitutes for Shaking Incubator Baths," *Enzyme* 45:67-70 (1991)) with octanoyl sphingosine and uridinediphospho[^{3}H]glucose (Shukla, G.S. et al., "Glucosylceramide Synthase of Mouse Kidney: Further Characterization and Improved Assay Method," *Arch. Biochem. Biophys.* 283:372-378 (1990)). The lipoidal substrate (85 μg) was added in liposomes made from 0.57 mg dioleoylphosphatidylcholine and 0.1 mg of Na sulfatide. Confluent cells were washed, then homogenized with a micro-tip sonicator at 0°C for 3 x 30 sec; ~0.2 mg of protein was used in each assay tube. In the case of the aromatic inhibitors, the test compound was simply evaporated to dryness from solution in the incubation tube. This method of adding the inhibitor was found to give the same results as addition as a part of the substrate liposomes. The aliphatic inhibitors, which appeared to be less soluble in water, were added as part of the substrate liposomes.

Acid and neutral ceramidases were assayed under conditions like those above, but the medium contained 110 μM [1-^{14}C]decanoyl sphingosine (10^5 cpm) in 340 μM dioleoylphosphatidylcholine liposomes and 0.34 mg of MDCK cellular protein homogenate. The acid enzyme was incubated in 32.5 mM citrate-Na^+ (pH 4.5) and the neutral enzyme buffer was 40 mM Tris-Cl^- (pH 7.1 at 37°C). After 60 min in the ultrasonic bath, 3 ml of C-M 2:1, carrier decanoic acid, and 0.6 ml of 0.9% saline were added and the lipids in the lower layer were separated by TLC with C-HOAc 9:1. The liberated decanoic acid was scraped off the glass plate and counted.

Ceramide synthase was assayed with 1 μM [3-^{3}H]sphingosine (70,000 cpm, repurified by column chromatography), 0.2 mM stearoyl-CoA, 0.5 mM dithiothreitol, and ~300 μg of MDCK homogenate protein in 25 mM phosphate-K^+ buffer, pH 7.4, in a total volume of 0.2 ml. The incubation (for 30 min) and TLC were carried out as above and the ceramide band was counted.

Sphingomyelin synthase was evaluated with 44 μM [^{14}C]decanoyl sphingosine (10^5 cpm) dispersed with 136 μM dioleoyllecithin as in th ceramide synthase assay, and 5 mM EDTA and 50 mM Hep s-Na^+ pH 7.5, in a total volume of 0.5 ml. MDCK homogenate was centrifuged at 600 X g briefly, then at 100,000 X g for 1 h, and the

pellet was suspended in water and sonicat d with a dipping probe. A portion of this suspension containing 300 μg of protein was used. Incubation was at 37°C for 30 min, after which the lipids were treated as above, using C-M-W 60:35:8 for the isolation of the labeled decanoyl SM.

Acid and neutral SMase assays were based on the procedures of Gatt et al. (Gatt, S. et al., "Assay of Enzymes of Lipid Metabolism With Colored and Fluorescent Derivatives of Natural Lipids," *Meth. Enzymol.* 72:351-375 (1981)), using liposomes containing NBD-SM dispersed like the labeled ceramide (10 μM substrate and 30 μM lecithin). The assay medium for the neutral enzyme also contained 50 mM Tris-Cl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.29 mg of MDCK cell protein in a total volume of 0.25 ml. Incubation was at 37°C for 30 min in the ultrasonic bath, then the fluorescent product, NBD-ceramide, was isolated by partitioning the assay mixture with 0.45 ml 2-propanol, 1.5 ml heptane, and 0.2 ml water. After centrifugation, a trace of contaminating NBD-SM was removed from 0.9 ml of the upper layer by washing with 0.35 ml water. The upper layer was analyzed with a fluorometer (460 nm excitation, 515 nm emission).

Acid SMase was assayed with the same liposomes in 0.2 ml of assay mixture containing 125 mM NaOAc (pH 5.0) and 61 μg of cell protein, with 60 min of incubation at 37°C. The resultant ceramide was determined as above.

Results

Table 1 lists the aromatic compounds (see Formula I) synthesized and their migration rates on silica gel TLC plates. Separation of the *threo*- and *erythro*-steroisomers by TLC was generally very good, except for BML-120, -121, and -122 in the acidic solvent. In the basic solvent BML-119 and BML-122 yielded poorly resolved double bands. BML-112 was unexpectedly fast-running, especially when compared with BML-120; both are presumably dihydrochlorides.

TABLE 1
Structures of the Aromatic Inhibitors

BML Number or Name	R Group	Phenyl Substituent	TLC hR_f Value[a]
PDMP[b]	morpholino		34(47)
PPMP	morpholino		(53)
112	N-phenylpiperazino		56
113	morpholino	*p*-fluoro	25

114	diethylamino		25
115	piperidino (pentamethyleneimino)		29
116	hexamethyleneimino		34
117[b]	morpholino	*p*-fluoro	41
118	piperidino	*p*-fluoro	26
119	pyrrolidino (tetramethyleneimino)		20-70(44)
120	1-methylpiperazino		7-62
121	3-dimethylaminopiperidino		1-30
122	N-methylethanolamino		6-71
123	azetidino (trimethyleneimino)		12
124	amino		15
125	morpholino	*p*-methoxy	37
126	pyrrolidino	*p*-methoxy	(50)

[a] Only the relative R_f value of the faster-moving band is shown. The first value was obtained with solvent A, the second with solvent C, and the numbers in parentheses, with solvent B. In the case of BML-117, -125, and -126, a 20-cm high TLC plate was used to improve the seperation.
[b] The fatty acid chain suggested by the R' group is decanoyl, not palmitoyl.

Table 2 describes four aliphatic inhibitors (see Formula II), which can be considered to be ceramide analogs in which the C-1 hydroxyl group is replaced by a cyclic amine. It should be noted that the carbon frameworks of compounds in Tables 1 and 2 are numbered differently (see Formulas I and II), thus affecting comparisons of stereochemical configurations. The *threo*- and *erythro*-isomers separated very poorly on TLC plates. Like the aromatic inhibitors, however, the morpholine compounds ran faster than the pyrrolidine compounds. The latter are presumably more strongly adsorbed by the silica gel because they are more basic.

TABLE 2
Characterization of the Sphingosyl Inhibitors

Number	R Group	Sphingol Structure	TLC hR_f Value[a]
IV-181A	morpholino	2R,3S	43
IV-206A	morpholino	2R,3R	40
IV-230A	pyrrolidino	2R,3S	31
IV-231B	pyrrolidino	2R,3R	31

[a] TLC solvent: C-M-HOAc 90:5:10. Similar but faster migrations were obtained with solvent A.

Structure-activity correlations. The results of testing the compounds in an assay system for GlcCer synthase are listed in Table 3. Each inhibition determination (± SD) shown in Table 3 was carried out in triplicate. Some of the inhibitors were tested as mixtures of DL-*erythro*- and DL-*threo*-isomers (see column 4). Only the D-*threo* enantiomer in each mixture was predicted to be the actual enzyme inhibitor (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987)); the content of this isomer was calculated by measuring the proportions of the *threo*- and *erythro*- racemic mixtures by quantitative TLC. The DL-*threo* contents were found to be in the range of 40 to 72%. The comparisons, in the case of the mixtures, are therefore approximate (most of the samples were not purified to remove the three less-active isomers and the observed data were not corrected for the level of the primary enantiomers). The separation of the *threo*- and *erythro*- forms is most conveniently accomplished by crystallization, but the specific conditions vary for each substance; thus only BML-119, a strong inhibitor, was separated into its *threo*- and *erythro*- forms. BML-112 is not included in Table 3 because it had no inhibitory activity against GlcCer synthase of rabbit liver microsomes.

TABLE 3
Inhibition of Ceramide Glucosyltransferase of MDCK cell Homogenates by Different Compounds

Inhibitor Number	% Inhibition at 80 μM	Inhibition at 5 μM	Active Isomer[h]
BML-113	60 ± 4.7[a]		29
BML-114	31 ± 2.9[a]		20
BML-115	84 ± 0.8[a] 82 ± 0.3[b]	12.4 ± 0.7[f]	27
BML-116	28 ± 3.2[a]		27
BML-117	35 ± 0.6[b]		36
BML-118	62 ± 0.4[b]	8.3 ± 1.4[f]	32
BML-119	94 ± 1.4[b] 97 ± 0.1[c] 96 ± 0.1[d]	51 ± 2.3[e] 49 ± 0.8[f]	29
BML-120	11 ± 3.0[c]		26
BML-121	11 ± 0.4[c]		28
BML-122	58 ± 1.6[d]		26
BML-123	86 ± 0.1[d]	15 ± 0.8[f]	33
BML-124	-2 ± 1.6[d]		15
BML-125		9 ± 3.0[e]	26
BML-126	60 ± 1.8[e]	54 ± 0.3[f]	34
PDMP	90 ± 0.8[a]	16 ± 1.8[f]	100
PPMP		32 ± 1.8[e] 32 ± 0.7[f]	100
IV-181A		12 ± 0.2[g]	100
IV-206A		73 ± 1.5[g]	100
IV-230A		19 ± 2.1[g]	100
IV-231B		87 ± 0.4[g]	100

[a-g] Different samples were assayed as parts of different experiments.
[h] Percent of the active D-stereoisomer in the synthesized sample, estimated by scanning the two stained bands, assuming the slower one was the (racemic) active form.

Comparison of PDMP (1R,2R-decanoate) and PPMP (1R,2R-palmitate), when evaluated at the same time in Expt. f, shows that an increase in the chain length of

the N-acyl group from 10 to 16 carbon atoms distinctly improved the inhibitory activity against GlcCer synthase, as noted before (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.* 111:191-196 (1992)). Accordingly, most of the other compounds were synthesized with the palmitoyl group for comparison with PPMP. The comparisons between the best inhibitors are clearer at the 5 μM level.

Replacing the oxygen in the morpholine ring of PPMP with a methylene group (BML-115) improved activity ~1.4-fold (calculated from the inhibitions at 5 μM in Expt. f and relative purities, and assuming that the percent inhibition is proportional to concentration in this region: 12.4/27 x 100/32 = 1.4). Previous comparison with mouse brain, human placenta, and human Gaucher spleen glucosyltransferase also showed that replacing the morpholino ring with the piperidino ring in a ketone analog of PDMP (1-phenyl-2-decanoylamino-3-piperidino-1-propanone) produced a much more active inhibitor (Vunnam, R.R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids* 26:265-278 (1980)).

Replacing the piperidine group with a 7-membered ring (BML-116) greatly decreased the activity, while use of a 5-membered ring (BML-119) quadrupled the effectiveness (50 vs 12.4% inhibition). A 4-membered ring (BML-123) yielded a compound about as effective as the piperidino compound. The parent amine (BML-124), its *N,N*-diethyl analog (BML-114), and the sterically bulky *N*-phenylpiperazine analog (BML-112) displayed little or no activity.

Replacing a hydrogen atom with a fluorine atom in the *p*-position of the phenyl ring decreased the inhibitory power (BML-117 vs PDMP and BML-118 vs BML-115). Substitution of the *p*-position with an electron-donating moiety, the methoxy group, had a similar weakening effect in the case of the morpholino compound (BML-125 vs PPMP). Comparison of the pyrrolidino compounds, which are more basic than the morpholino compounds, showed that the methoxy group enhanced the inhibitory power (BML-126 vs BML-119).

Preparations of BML-119 were separated into *threo* and *erythro* racemic mixtures by HPLC on a Waters Microbondapak C_{18} column, using M-W-conc. NH_4OH 90:10:0.2 as the lution solvent. The material eluting arlier (but migrating more slowly on a TLC plate) was call d BML-130; the later eluting material (fast r by TLC) was called BML-129. Assay of GlcCer synthase with each preparation at 5 μM showed 15% inhibition by BML-129 and 79% inhibition by BML-130. TLC analysis of

the two preparations rev aled incomplete separation, which could explain the minor inhibition by BML-129. When the two stereoisomers were separated by preparative TLC, the difference in effectiveness was found to be somewhat higher, evidently due to the better separation by this method. Thus the slower-migrating stereoisomer accounted for all or nearly all of the inhibitory activity, as noted with PDMP (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987)).

Comparison of the two pairs of aliphatic inhibitors (bottom of Table 3) showed that the 2R,3R (D-*threo*) form is the primary inhibitor of glucosyltransferase. This finding is in agreement with previous identification of the active PDMP isomer as being the D-*threo* enantiomer. However, unlike the aromatic analog, BML-129 (2R,3S/2S,3R), there was a relatively small but significant activity in the case of the (*erythro*) 2R,3S stereoisomer. The *erythro* form of PDMP was found to inhibit cell proliferation of rabbit skin fibroblasts almost as well as R,R/S,S-PDMP but it did not act on the GSLs (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem. (Tokyo)* 108:525-530 (1990)). As noted with the aromatic analogs, the pyrrolidine ring was more effective than the morpholine ring (Table 3).

Comparison of the aliphatic and corresponding aromatic inhibitors can be made in the case of the optically active morpholine compounds PPMP and IV-206A, both of which have the R,R structure and the same fatty acid. Here it appears that the aliphatic compound is more effective (Table 3). However in a second comparison, at lower concentrations with the inhibitors incorporated into the substrate liposomes, the degree of inhibition was 77 ± 0.9% with 3 μM IV-231B and 89 ± 0.6% with 6 μM DL-*threo* BML-119.

Evaluations of cultured cell growth. Exposure of five different cancer cell lines to inhibitors at different concentrations for 4 or 5 days showed that the six BML compounds most active against GlcCer synthase were very effective growth inhibitors (Table 4). The IC_{50} values (rounded off to one digit in the table) ranged from 0.7 to 2.6 μM.

TABLE 4
Inhibition of Tumor Cell Growth *In Vitro* by Various Inhibitors

Cell Type	BML-115	BML-118	BML-119	BML-123	BML-126	BML-129	BML-130
MCF-7	2	2	2	2	1	3	2
H-460	2	2	1	1	1	2	3
HT-29	2		1	2	1	2	2
9L	2	2	1	2	2	2	2
UMSCC-10A	1		1	1	1	2	2

Figure 1 shows growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors, as described above. The BML compounds were used as synthesized (mixtures of DL-*threo* and -*erythro* stereoisomers) while the PDMP and PPMP were optically resolved R,R isomers. The concentrations shown are for the mixed racemic stereoisomers, since later work (Table 4) showed that both forms were very similar in effectiveness. Figure 1 illustrates the relatively weak effectiveness of R,R-PPMP and even weaker effectiveness of R,R-PDMP. The three new compounds, however, are much better inhibitors of GlcCer synthase and growth. These differences in growth inhibitory power correlate with their effectiveness in MDCK cell homogenates as GlcCer synthase inhibitors. Some differences can be expected due to differences in sensitivity of the synthase occurring in each cell type (the synthases were assayed only in MDCK cells).

Growth inhibition by each of the most active BML compounds occurred in an unusually small range of concentrations (*e.g.*, the slopes of the cytotoxic regions are unusually steep). Similar rapid drop-offs were seen in another series of tests with 9L cells, in which BML-119 yielded 71% of the control growth with 1 μM inhibitor, but only 3% of control growth with 3 μM. Growth was 93% of control growth with 2 μM BML-130 but only 5% of controls with 3 μM inhibitor. While some clinically useful drugs also show a narrow range of effective concentrations, this is a relatively uncommon relationship.

When the *erythro*- and *threo*-stereoisomeric forms of BML-119 (-129 and -130) were compared, they were found to have similar effects on tumor cell growth (Table 4). This observation is similar to the results with PDMP isomers in fibroblasts cited

above (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem. (Tokyo)* 108:525-530 (1990)). Since enzymes are optically active and since stereoisomers and enantiomers of drugs can differ greatly in their effect on enzymes, it is likely that BML-129 and BML-130 work on different sites of closely related metabolic steps.

Figure 2 shows the amount of cellular protein per dish for MDCK cells cultured for 24 h in medium containing different concentrations of the separated *erythro-* and *threo-* isomers of BML-119, as percent of the incorporation by cells in standard medium. Each point shown in Figure 2 is the average of values from three plates, with error bars corresponding to one standard deviation.

Figure 3 shows [^{3}H]thymidine incorporation into DNA of MDCK cells incubated as in Figure 2. The values in Figure 3 are normalized on the basis of the protein content of the incubation dishes and compared to the incorporation by cells in standard medium.

Figures 2 and 3 thus provide comparison of the two stereoisomers with MDCK cells. The isomers were found to inhibit growth and DNA synthesis with similar effectiveness. Thus the MDCK cells behaved like the human tumor cells with regard to IC_{50} and the narrow range of concentrations resulting in inhibition of protein and DNA synthesis.

Surprisingly, the aliphatic inhibitor IV-231B exerted no inhibitory effect on MDCK cell growth when incubated at 20 μM for 1 day or 1 μM for 3 days. Tests with a longer growth period, 5 days, in 5 μM inhibitor also showed no slowing of growth. The dishes of control cells, which contained BSA as the only additive to the medium, contained 3.31 $\pm$ 0.19 mg of protein, while the IV-231B/BSA treated cells contained 3.30 $\pm$ 0.04 mg.

Lipid changes induced in the cells. Examination by TLC of the alkali-stable MDCK lipids after a 24 h incubation disclosed that BML-130 was more effective than BML-129 in lowering GlcCer levels, as expected from its greater effectiveness in vitro as a glucosyltransferase inhibitor. The level of GlcCer, estimated visually, was greatly lowered by 0.3 μM BML-130 or 0.5 μM BML-129. The levels of the other lipids visible on the plate (mainly sphingomyelin (SM), cholesterol, and fatty acids) were changed little or not at all. BML-129 and the GlcCer synthas inhibitor, BML-130, were readily detect d by TLC at the various levels used, showing that they were taken up by the cells during the incubation period at dose-dependent rates. Lactosylceramide

overlapped the inhibitor bands with solvent D but was well separated with solvent E, which brought the inhibitors well above lactosylceramide.

Ceramide accumulation was similar for both stereoisomers (data not shown). An unexpected finding is that noticeable ceramide accumulation appeared only at inhibitor concentrations that were more than enough to bring GlcCer levels to a very low point (*e.g.*, at 2 or 4 μM). The changes in ceramide concentration were quantitated in a separate experiment by the diglyceride kinase method, which allows one to also determine diacylglycerol (DAG) concentration (Preiss, J.E. et al., "Quantitative Measurement of SN-1,2-Diacylglycerols Present in Platelets, Hepatocytes, and Ras- and Sis-Transformed Normal Rat Kidney Cells," *J. Biol. Chem.* 261:8597-8600 (1986)). The results (Table 5) are similar to the visually estimated ones: at 0.4 μM BML-129 or -130 there was little effect on ceramide content but at 4 μM inhibitor, a substantial increase was observed. (While the duplicate protein contents per incubation dish were somewhat erratic in the high-dose dishes, in which growth was slow, the changes were nevertheless large and clear.) Accumulation of ceramide had previously been observed with PDMP, at a somewhat higher level of inhibitor in the medium (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). From the data for cellular protein per incubation dish, it can be seen that there was no growth inhibition at the 0.4 μM level with either compound but substantial inhibition at the 4 μM level, especially with the glucosyltransferase inhibitor, BML-130. This finding is similar to the ones made in longer incubations with human cancer cells.

TABLE 5
Effects of BML-129 and -130 on MDCK Cell Growth and the Content of Ceramide and Diacylglycerol

Growth Medium	Protein	Ceramide	Diglyceride
	μg/dish	nmol/mg protein	
Controls	490 560	1.04 0.96	4.52 5.61
0.4 μm BML-129	500 538	1.29 0.99	5.51 5.13
0.4 μm BML-130	544 538	0.94 0.87	4.73 5.65
4 μm BML-129	396 311	3.57 3.78	9.30 9.68

4 μm BML-130	160 268	5.41 3.34	11.9 8.71

In a separate study of ceramide levels in MDCK cells, BML-130 at various concentrations was incubated with the cells for 24 h. The ceramide concentration, measured by TLC densitometry, was 1.0 nmol/mg protein at 0.5 μM, 1.1 at 1 μM, 1.5 at 2 μM, and 3.3 at 4 μM. The results with BML-129 were virtually identical.

It is interesting that the accumulation of ceramide paralleled an accumulation of diacylglycerol (DAG), as observed before with PDMP (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). DAG is ordinarily considered to be an activator of protein kinase C and thus a growth stimulator, but the low level of GlcCer in the inhibited cells may counteract the stimulatory effect. Ceramide reacts with lecithin to form SM and DAG, so it is possible that the increased level of the latter reflects enhanced synthesis of the phosphosphingolipid rather than an elevated attack on lecithin by phospholipase D. Arabinofuranosylcytosine (ara-C), an antitumor agent, also produces an elevation in the DAG and ceramide of HL-60 cells (Strum, J.C. et al., "1-β-D-Arabinofuranosylcytosine Stimulates Ceramide and Diglyceride Formation in HL-60 Cells," *J. Biol. Chem.* 269:15493-15497 (1994)).

TLC of MDCK cells grown in the presence of 0.02 to 1 μM IV-231B for 3 days showed that the inhibitor indeed penetrated the cells and that there was a great depletion of GlcCer, but no ceramide accumulation. The depletion of GlcCer was evident even at the 0.1 μM level and virtually no GlcCer was visible at the 1 μM level; however the more polar GSLs were not affected as strongly. After incubation for 5 days in 5 μM inhibitor, all the GSLs were virtually undetectable. The ceramide concentrations in the control and depleted cells were very similar: 13.5 ± 1.4 vs 13.9 ± 0.2 μg/mg protein.

The lack of ceramide accumulation in cells exposed to the aliphatic inhibitors was examined further to see if it might be due to differential actions of the different inhibitors on additional enzymes involving ceramide metabolism. For example, IV-231B might block ceramide synthase and thus prevent accumulation despite the inability f th cells to utilize ceramide for GlcCer synthesis. However, assay of ceramid synthase in homogenized cells showed it was not significantly affected by

5 μM inhibitors (Table 6). There did appear to be moderate inhibition at the 50 μM lev l with PDMP and the aliphatic inhibitor.

TABLE 6
Effect of Inhibitors on Acid and Neutral Ceramidases and Ceramide Synthase of MDCK Cells

Inhibitor Tested	Enzyme Activity (% of control)		
	Ceramidase pH 4.5	Ceramidase pH 7.4	Ceramide Synthase
D-*threo*-PDMP, 5 μM	97 ± 4	116 ± 19	99 ± 5
D-*threo*-PDMP, 50 μM	133 ± 13[a]	105 ± 11	66 ± 9[a]
BML-129, 5 μM	108 ± 8	100 ± 0	97 ± 0
BML-129, 50 μM	171 ± 26[a]	99 ± 2	102 ± 1
BML-130, 5 μM	107 ± 11	100 ± 15	108 ± 10
BML-130, 50 μM	160 ± 21[a]	100 ± 15	106 ± 29
IV-231B, 5 μM	106 ± 3	116 ± 20	90 ± 8
IV-231B, 50 μM	113 ± 8	112 ± 3	71 ± 18[a]

[a] Notable differences.

Assay of the two kinds of ceramidase (Table 6) showed that there was no effect of either the aliphatic or aromatic inhibitors at the 5 μM level, at which point cell growth is completely stopped in the case of the pyrrolidino compounds. At the 50 μM level, however, the acid enzyme was stimulated markedly by the aromatic inhibitors, particularly the two stereoisomeric forms of the pyrrolidino compound.

Sphingomyelin synthase was unaffected by PDMP or the aliphatic inhibitor but BML-129 and -130 produced appreciable inhibition at 50 μM (54% and 61%, respectively) (Table 7).

TABLE 7
Effect of Inhibitors n Acid and Neutral Sphingomy linases and Sphingomy lin Synthase

Inhibitor Tested	Enzyme Activity (% of control)		
	Sphingomyelinase pH 4.5	Sphingomyelinase pH 7.1	Sphingomyelinase Synthase[a]
D-*threo*-PDMP, 5 μM	102 ± 3	121 ± 13	
D-*threo*-PDMP, 50 μM	100 ± 3	108 ± 8	
BML-129, 5 μM	108 ± 4	105 ± 11	84 ± 27
BML-129, 50 μM	97 ± 3	142 ± 11[b]	46 ± 11[b]
BML-130, 5 μM	109 ± 1	110 ± 7	87 ± 14
BML-130, 50 μM	114 ± 2	152 ± 14[b]	39 ± 18[b]
IV-231B, 5 μM	101 ± 7	131 ± 3[b]	
IV-231B, 50 μM	112 ± 11	120 ± 3[b]	

[a] Data for PDMP and IV-231B are not shown here as they were tested in other experiments; no effect was seen.
[b] Notable differences.

Neutral sphingomyelinase (SMase) was distinctly stimulated by the aliphatic inhibitor, IV-231B, even at 5 μM (Table 7). From this one would expect that the inhibitor would produce accumulation of ceramide, yet it did not. The two pyrrolidino compounds produced appreciable stimulation at the 50 μM level. No significant effects were obtained with acid SMase.

Discussion

The present invention shows that the nature and size of the tertiary amine on ceramide-like compounds exerts a strong influence on GlcCer synthase inhibition, a 5-membered ring being most active. It also shows that the phenyl ring used previously to simulate the trans-alkenyl chain corresponding to that of sphingosine could, with benefit, be replaced with the natural alkenyl chain.

Findings with the most active GlcCer synthase inhibitors in growth tests compare favorably with evaluations of some clinically useful chemotherapeutic agents on three of the tumor cell lines in the same Drug Evaluation Core Laboratory. The IC_{50} values were 0.2 to 6 μM for cisplatin, 0.02 to 44 μM for carboplatin, 0.03 to 0.2 μM for methotrexate, 0.07 to 0.2 μM for fluorouracil, and 0.1 to 1 μM for etoposide. Unlike

these agents, the compounds of the present invention yielded rath r similar ffects with all the cell types, including MDCK cells, and thus hav wider potential chemotherapeutic utility. This uniformity of action is consistent with the idea that GSLs play a wide and consistent role in cell growth and differentiation.

An important observation from the MDCK cell study is that strong inhibition of cell growth and DNA synthesis occurred only at the same concentrations of aromatic inhibitor that produced marked ceramide accumulation. This observation supports th assertion that ceramide inhibits growth and enhances differentiation or cell death (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters* 307:211-214 (1992)). It also agrees with previous work with octanoyl sphingosine, a short chain ceramide that produced greatly elevated levels of natural ceramide and slowed growth (Abe, A. et al., "Metabolic Effects of Short-Chain Ceramide and Glucosylceramide on Sphingolipids and Protein Kinase C," *Eur. J. Biochem.* 210:765-773 (1992)). It is also in agreement with a finding that some synthetic, nonionic ceramide-like compounds did not inhibit GlcCer synthase even though they behave like ceramide in blocking growth (Bielawska, A. et al., "Ceramide-Mediated Biology. Determination of Structural and Stereospecific Requirements Through the Use of N-Acyl-Phenylaminoalcohol Analogs," *J. Biol. Chem.* 267:18493-18497 (1992)). Compounds tested included 20 μM D-*erythro*-N-myristoyl-2-amino-1-phenyl-1-propanol, its L-enantiomer, the four stereoisomers of *N*-acetylsphinganine, and *N*-acetylsphingosine. Furthermore, the lack of growth inhibition and ceramide accumulation in cells treated with the aliphatic inhibitor IV-231B is also consistent with the correlation between ceramide level and growth rate.

The accumulation of ceramide that occurred at higher levels of GlcCer synthase inhibitors could be attributed not only to blockage of ceramide utilization, but also to blockage of SM synthesis or ceramide hydrolase. This possibility is especially relevant to the R,S-, S,R-, and S,S-isomers, which seem to exert effects on sphingolipids without strongly inhibiting GlcCer synthesis. The tests with both the DL-*erythro*-pyrrolidino inhibitor (BML-129) and the DL-*threo*-pyrrolidino inhibitor (BML-130), at a level producing strong growth inhibition, showed that neither material at a low concentration inhibited the enzymes tested *in vitro* (Tabl s 6 and 7) but they did caus growth inhibition as well as accumulation of ceramide. PDMP, at relatively high concentrations (50 μM), was found to inhibit SM synthas in growing CHO cells (Rosenwald, A.G. t al., "Effects of a Sphingolipid Synthesis Inhibitor on Membrane

Transport Through the Secretory Pathway," *Biochemistry* 31:3581-3590 (1992)). In the test with MDCK homogenates, it did not inhibit this synthase, in agre ment with the finding that labeled palmitate incorporation into SM was stimulated by PDMP (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)).

Retinoic acid is a growth inhibitor of interest in cancer chemotherapy and a possible adjunct in the use of the inhibitors of the present invention. It has been found to elevate ceramide and DAG levels (Kalén, A. et al., "Elevated Ceramide Levels in GH4C1 Cells Treated with Retinoic Acid," *Biochim. Biophys. Acta* 1125:90-96 (1992)) and possibly lower lecithin content (Tang, W. et al., "Phorbol Ester Inhibits 13-Cis-Retinoic Acid-Induced Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate in Cultured Murine Keratinocytes: a Possible Negative Feedback Via Protein Kinase C-Activation," *Cell Bioch. Funct.* 9:183-191 (1991)).

D-*threo*-PDMP was found to be rather active in delaying tumor cell growth or in producing complete cures in mice (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.* 38:23-30 (1987)) but high doses were needed. From the data in Figure 1, the inhibitors of the present invention are approximately 30 times as active, so the dosage levels are typical of clinically useful drugs. The need to use high doses with PDMP was attributed to rapid inactivation by cytochrome P450 (Shukla, A. et al., "Metabolism of D-[^{3}H]PDMP, an Inhibitor of Glucosylceramide Synthesis, and the Synergistic Action of an Inhibitor of Microsomal Monooxygenase," *J. Lipid Res.* 32:713-722 (1991)). Cytochrome P450 can be readily blocked by various nontoxic drugs such as cimetidine, therefore high levels of the compounds of the present invention can be maintained.

SPECIFIC EXAMPLE 2

A series of inhibitors based on substitutions in the phenyl ring of P4 were synthesized and studied. It was found that the potency of the inhibitors in blocking GlcCer synthase was mainly dependent upon hydrophobic and electronic properties of the substituent. Surprisingly, a linear relationship was found between log [IC_{50}] and hydrophobic parameter (π) + electronic parameter (δ). This correlation suggested that electron donating and hydrophilic characters of the substituent nhance the potency as an inhibitor. This observation resulted in the synthesis of novel compounds that are more active in blocking glucosylceramide formation. Two compounds, dioxy D-*t*-P4 compounds, D-*t*-3',4'-ethylenedioxy-P4 and D-t-4'-hydroxy-P4, were observed to

be significantly more potent than other tested inhibitors. In particular, at 11.3 nM D-*t*-3',4'-ethylenedioxy-P4, 80% of glucosylceramide in MDCK cell was depleted without any ceramide accumulation and cell growth inhibition. The potency of D-*t*-3',4'-ethylenedioxy-P4 appears to be not only regulated by hydrophobic and electronic properties but also by stearic properties of the substituents on the phenyl group.

Materials and Methods

Materials. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, MO., Lancaster Synthesis Inc., Windham, NH. and Maybridge Chemical Co., Cornwall, UK. Silica gel for column chromatography (70-230 mesh ASTM) and Silica gel thin layer chromatography plates were purchased from Merck Co. The reagents and their sources were: non-hydroxy fatty acid ceramide from bovine brain and delipidated bovine serum albumin (BSA) from Sigma; dioleoyphosphatidylcholine from Avanti; DL-dithiothreitol from Calbiochem; 1-[^{3}H]-glucose uridine diphosphate from NEN. Octanoylsphingosine, glucosylceramide and sodium sulfatide were prepared as previously described. Abe, A. et al., *Eur. J. Biochemistry* 210:765-773 (1992).

General synthesis of inhibitors. The aromatic inhibitors were synthesized by the Mannich reaction from 2-N-acylaminoacetophenone, paraformaldehyde, and pyrrolidine, and then the reduction from sodium borohydride as described before. Inokuchi, J. et al., *J. Lipid. Res.* 28:565-571 (1987); Abe, A. et al., *J. Lipid. Res.* 36:611-621 (1995). The reaction produces a mixture of four isomers, due to the presence of two asymmetric centers. For these syntheses in which phenyl-substituted starting materials were used, the chloro, methoxy, methylenedioxy, methyl groups in the acetophenone structure were brominated and converted to the primary amine. Bromation of the methoxyacetophenone, dimethyoxyacetophenone, 3',4'-(methylenedioxy)acetophenone were performed in chloroform at room temperature and recrystallized from ethyl acetate and hexane.

Synthesis of 1-(4'-hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol. The synthesis of 1-(4'-hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol is described in detail in Figure 8. This synthesis differs from those of the other compounds because of the need for the placement of a protecting group on the free hydroxyl (step 1) and its subsequent removal (step 7). All other syntheses employ a similar synthetic scheme (st ps 2 to 6).

4'-B nzyloxyacetoph non formation (step 1): 4'-Hydroxyacetoph none (13.62 g, 100 mmol), benzylbromide (17.1g, 100 mmol), and cesium carbonat (35.83 g, 100 mmol) were added to tetrahydrofuran at room temperature and stirred

overnight. The product was concentrated to dryness and recrystallized from ether and hexane to yield 15 g of 4'-benzyloxyacetophenone which appeared as a white powder. An R_f of 0.42 was observed when resolved by thin layer chromatography using methylene chloride. ^1H nmr (δ, ppm, $CDCl_3$), 7.94 (2H, δ, 8.8 Hz, O-Ar-C(O)), 7.42 (5H, m, Ar'CH_2O-), 7.01 (2H, δ, 8.8 Hz, O-Ar-C(O)), 5.14 (2H, s, Ar'CH_2O-), 2.56 (3H, S, CH_3).

Bromination of 4'-benzyloxyacetophenone (step 2): Bromine (80 mmol) was added dropwise over 5 min to a stirred solution of 4'-benzyloxyacetophenone (70 mmol) in 40 ml chloroform. This mixture was stirred for an additional 5 min and quenched with saturated sodium bicarbonate in water until the pH reached 7. The organic layers were combined, dried over $MgSO_4$, and concentrated to dryness. The crude mixture was purified over a silica gel column and eluted with methylene chloride to yield 2-bromo-4'-benyloxyacetophenone. An R_f of 0.62 was observed when resolved by thin layer chromatography using methylene chloride. 1H nmr (δ, ppm, $CDCl_3$), 7.97 (2H, δ, 9.2 Hz, O-Ar-C(O)), 7.43 (5H, m, Ar'CH_2O-), 7.04 (2H, δ, 9.0 Hz, O-Ar-C(O)), 5.15 (2H, s, Ar'CH_2O-), 4.40 (2H, s, CH_2Br).

2-Amino-4'-benzyloxyacetophenone HCl formation (step 3): Hexamethylenetetramine (methenamine, 3.8 g, 23 mmol) was added to a stirred solution of 2-bromine-4'-benyloxyacetophenone (6.8 g, 23 mmol) in 100 ml chloroform. After 4 h the crystalline adduct was filtered and washed with chloroform. The product was dried and heated with 150 ml methanol and 8 ml of concentrated HCl in an oil bath at 85°C for 3 h. Upon cooling the precipitated hydrochloride salt (2.5 g) was removed by filtration. The filtrate was left at -20°C overnight and additional product (2.1 g) was isolated. The yield was 4.6 g (82.6%). $[M^+H]^+$: 242 for $C_{15}H_{16}NO_2$. ^1H nmr (δ, ppm, $CDCl_3$), 8.38 (2H, bs, NH_2), 7.97 (2H, δ, 8.8 Hz, O-Ar-C(O)), 7.41 (5H, m, Ar'CH2O-), 7.15 (2H, δ, 8.6 Hz, O-Ar-C(O)), 5.23 (2H, s, Ar'CH_2O-), 4.49 (2H, s, CH_2NH_2).

2-Palmitoylamino-4'-benyloxyacetophenone formation (step 4): Sodium acetate (50% in water, 29 ml) was added in three portions to a stirred solution of 2-amino-4'-benzyloxyacetophenone HCl (4.6 g, 17 mmol) and tetrahydrofuran (200 ml). Palmitoyl chloride (19 mmol) in tetrahydrofuran (25 ml) was added dropwise over 20 min yielding a dark brown solution. The mixture was stirred ov might at room temperature. The aqueous fraction was remov d by use of a separatory funnel and chloroform/methanol (2/1, 150 ml) was added to the organic layer which was then washed with water (50 ml). The yellow aqueous layer was extracted once with

chloroform (50 ml). The organic solutions were then pooled and rotoevaporated until near dryness. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was cooled to 4°C for 2 h. The crystals were filtered and washed with cold hexane and dried in a fume hood overnight. The product yield was 3.79 g (8 mmol). An R_f of 0.21 was observed when resolved by thin layer chromatography using methylene chloride. $[M^+H]^+$: 479 for $C_{31}H_{45}NO_3$. ^1H nmr (δ, ppm, $CDCl_3$), 7.96 (2H, δ, 8.8 Hz, O-Ar-C(O)), 7.40 (5H, m, $Ar'CH_2O$-), 7.03 (2H, δ, 8.8 Hz, O-Ar-C(O)), 6.57 (1H, bs, NH_2), 5.14 (2H, s, $Ar'CH_2O$-), 4.71 (2H, s, $C(O)CH_2NHC(O)$), 2.29 (2H, t, 7.4 Hz, $C(O)CH_2(CH_2)_{13}CH_3$), 1.67 (2H, m, $C(O)CH_2(CH_2)_{13}CH_3$), 0.87 (3H, t, 6.7 Hz, $C(O)CH_2(CH_2)_{13}CH_3$).

1-(4'-Benzyloxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol formation (steps 5 and 6): 2-Palmitoylamino-4'-benyloxyacetophenone (3.79 g, 8.0 mmol), paraformaldehyde (0.25 g, 2.7 mmol), pyrrolidine (0.96 ml, 11.4 mmol) and ethanol (70 ml) were stirred under nitrogen. Concentrated HCl (0.26 ml) was added through the condensor and the mixture was heated to reflux for 16 h. The resultant brown solution was cooled on ice and then sodium borohydride (1.3 g, 34 mmol) was added in three portions. The mixture was stirred at room temperature overnight, and the product was dried in a solvent evaporator. The residue was redissolved in dichloromethane (130 ml) and hydrolyzed with 3N HCl (pH~4). The aqueous layer was extracted twice with dichloromethane (50 ml). The organic layers were pooled and washed twice with water (30 ml), twice with saturated sodium chloride (30 ml), and dried over anhydrous magnesium sulfate. The dichloromethane solution was rotoevaporated to a semisolid and purified by use of a silica rotor using a solvent consisting of 10% methanol in dichloromethane. This yielded a mixture of DL-*threo*- and DL-*erythro* enantiomers (2.53 g, 4.2 mmol). An R_f of 0.43 for the *erythro* diastereomers and 0.36 for the *threo* diastereomers was observed when resolved by thin layer chromatography using methanol:methylene chloride (1:9). $[M^+H]^+$: 565 for $C_{36}H_{56}N_2O_3$.

1-(4'-Hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol formation (step 7): A suspension of 20% Pd/C (40 mg) in acetic acid (15 ml) was stirred at room temperature under a hydrogen balloon for 15 min. 1-(4'-Benzyloxy)phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (420 mg, 0.74 mmol) was add d and the solution was stirred overnight. The suspension was filtered through a glass frit, and the filter was rinsed with acetic acid:methylene chloride (1:1, 5 ml). The filtrate was concentrated in vacuo and crystallized to yield a pale yellow semisolid (190 mg, 0.4

mmol). An R_f of 0.21 was bserved when resolved by thin layer chromatography using methanol:methylene chloride (1:9). $[M^+H]^+$: 475 for $C_{29}H_{50}N_2O_3$. ^1H nmr (δ, ppm, $CDCl_3$), 7.13 (4H, m, ArCHOH-), 7.14 (1H, δ, 6.9 Hz, -NH-), 5.03 (1H, δ, 3.3 Hz, CHOH-), 4.43 (1H, m, c-$(CH_2CH_2)_2NCH_2CH$), 3.76 (2H, m, c-$(CH_2CH_2)_2N$-), 3.51 (1H, m, c-$(CH_2CH_2)_2NCH_2$-), 3.29 (1H, m, c-$(CH_2CH_2)_2NCH_2$-), 2.97 (3H, m, c-$(CH_2CH_2)_2N$- and ArC(OH)H-), 2.08 (6H, m, -$C(O)CH_2(CH_2)_{13}CH_3$ and c-$(CH_2CH_2)_2N$-, 1.40 (2H, m, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.25 (2H, m, -$C(O)CH_2CH_2(CH_2)_{12}CH_3$), 0.87 (3H, t, 6.7 Hz, $C(O)CH_2(CH_2)_{13}CH_3$).

Synthesis of D-threo-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

2-Amino-3',4'-(ethylenedioxy)acetophenone HCl: Hexamethylenetetramine (methenamine, 5.4 g, 0.039 mol) was added to a stirred solution of phenacylbromide (10.0 g, 0.039 mol) in 200 ml chloroform. After 2 h, the crystalline adduct was filtered and washed with chloroform. The product was then dried and heated with methanol (200 ml) and concentrated HCl (14 ml) in an oil bath at 85°C for 2 h. On cooling, the precipitated ammonium chloride was removed by filtration and the filtrate was left in a freezer overnight. After filtration the crystallized phenacylamine HCl was washed with cold isopropanol and then with ether. The yield of this product was ~7.1 g (81%).

2-Palmitoylamino-3',4'-(ethylenedioxy)acetophenone: Aminoacetophenon HCl (7.1 g, 31 mmol) and tetrahydrofuran (300 ml) were placed in a 1 liter three-neck round bottom flask with a large stir bar. Sodium acetate (50% in water, 31 ml) was added in three portions to this suspension. Palmitoyl chloride (31 ml, 10 % excess, 0.036 mol) in tetrahydrofuran (25 ml) was then added dropwise over 20 min to yield a dark brown solution. This mixture was then stirred for an additional 2 h at room temperature. The resultant mixture was poured into a separatory funnel to remove the aqueous solution. Chloroform/methanol (2/1, 150 ml) was then added to the organic layer and washed with water (50 ml). The yellow aqueous layer was extracted once with chloroform (50 ml). The organic solutions were pooled and rotoevaportated until almost dry. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was then cooled to 4°C for 2 h. The crystals were filtered and washed with cold hexane until they were almost white and then dried in a fume hood overnight. The yield of the product was 27 mmol (11.6 g).

D-threo-1-(3',4'-ethyl n dioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propan l: almitoylaminoacetophenone (11.6 g, 0.027 mol), paraformaldehyde (0.81 g, 0.009 mol), pyrrolidine (3.6 ml, 0.042 mol) and thanol (250 ml) were added to a

500 ml round flask under nitrogen flow. Concentrat d HCl (0.8 ml) was added to this mixture through the reflux condenser and the mixture was refluxed for 16 h. The brown solution was cooled in an ice-bath. Sodium borohydride (2.28 g, 0.06 mol) was added in three portions. This mixture was stirred at room temperature for 3 h and then rotoevaporated. The residue was dissolved in 130 ml of dichloromethane and the borate complex hydrolyzed with HCl (3N) until the pH was approximately 4. The aqueous layer was extracted twice with 50 ml dichloromethane. The organic layers were pooled and washed twice with H_2O (30 ml), saturated NaCl (30 ml) and dried over anhydrous $MgSO_4$. The dichloromethane solution was rotoevaporated to a viscous oil which was purified by use of a Chromatotron with a solvent consisting of 10% methanol in dichloromethane to obtain a mixture of DL-*threo* and *erythro* enantiomers (2.24 g, 0.004 mol).

Resolution of inhibitor enantiomers. High performance liquid chromatography (HPLC) resolution of the enantiomers of DL-*threo* and DL-*erythro* are performed using a preparative HPLC column (Chirex 3014: [(S)-val-(R)-1-(a-naphtyl)ethylamine, 20 x 250 mm: Phenomenex], eluted with hexane-1,2-dichloroethane-ethanol-trifluroacetic acid 64:30:5.74:0.26, at a flow rate of 8 ml/min. The column eluent was monitored at 254 nm in both the preparative and analytical modes. Isolated products were reinjected until pure by analytical HPLC analysis, determined using an analytical Chirex 3014 column (4.6 x 250 mm) and the above solvent mixture at flow rate of 1 ml/min.

Glycosylceramide synthase activity. The enzyme activity was measured by the method previously described in Skukla, G. et al., *Biochim. Biophys. Acta* 1083:101-108 (1991). MDCK cell homogenate (120 μg of protein) was incubated with uridinediphosphate [^{3}H]glucose (100,000 cpm) and liposomes consisting of 85 μg octanoylsphingosine, 570 μg dioleoyphosphatidylcholine and 100 μg sodium sulfatide in 200 μl of reaction mixture and kept for 1 h at 37 °C. P4 and P4 derivatives dissolved in dimethyl sulfoxide were dispersed into the reaction mixture after adding liposomes. The final concentration of dimethyl sulfoxide was kept 1% under which the enzyme activity was not at all inhibited.

Cell culture and lipid extraction. One half million of MDCK cells were seeded into 10 cm style dish containing 8 ml serum free DMEM supplemented medium. Shayman, J.A. et al., *J. Biol. Chem.* 265:12135-12138 (1990). After 24 h the medium was replaced with 8 ml of th medium containing 0, 11.8, 118 or 1180 nM D-*t*-P4, D-*t*-3',4'-ethylenedioxy-P4 or D-4'-hydroxy)-P4. The GlcCer synthase

inhibitors were added into th medium as a one to one complex with delipidated BSA. Abe, A. et al., *J. Lipid. Res.* 36:611-621 (1995); Abe, A. et al., *Biochim. Biophys. Acta* 1299:331-341 (1996). The cells were incubated for 24 h or 48 h with the inhibitors. After the incubation, the cells were washed twice with 8 ml of cold PBS and fixed with 2 ml of cold methanol. The fixed cells were scraped and transferred to a glass tube. Another one ml of methanol was used to recover the remaining cells in the dish.

Three ml of chloroform was added to the tube and briefly sonicated using a water bath type sonicator. After centrifugation at 800g for 5 min, the supernatant was transferred into another glass tube. The residues were reextracted with chloroform/methanol (1/1). After the centrifugation, the resultant supernatant was combined with the first one. The residues were air-dried and kept for protein analysis. Adding 0.9% NaCl to the supernatant combined, the ratio of chloroform/methanol/aqueous was adjusted to 1/1/1. After centrifugation 800g for 5 min, the upper layer was discarded. Methanol/water (1/1) with the same amount of volume of the lower layer was used to wash. The resultant lower layer was transferred into a small glass tube and dried down under a stream of nitrogen gas. A part of the lipid was used for lipid phosphate determination. Ames, B.N., *Methods Enzymol.* 8:115-118 (1966). The remainder was analyzed using HPTLC (Merck).

Results

Synthesis of P4 and P4 derivatives. The preparation of P4 derivatives utilized the Mannich reaction from 2-N-acylaminoacetophenone, paraformaldehyde, and pyrrolidine, and then the reduction of DL-pyrrodino ketone from sodium borohydride. In most cases, no isolation of DL-pyrrodino ketones were performed to maintain solubility. The overall yields of the DL-*threo* and DL-*erythro* syntheses were ~ 10-30%. These derivatives were purified by the either silica gel column or rotors with solvent 5-12% methanol in dichloromethane to optimize the separation from the chiral column. To obtain the best separation, each injection contains no more than 150 mg, and fractions were pooled to obtain sufficient quantity of isomer of D-*threo* for further biological characterization.

Resolution of PDMP homologues by chiral chromatography. The structures of the parent compound, D-*threo*-P4 and the phenyl-substituted homologues including the new dioxy-substituted and 4'-hydroxy-P4 homologu s are shown in Figure 9. Initially the effect of each P4 isomer s parated by chiral chromatography on GlcCer synthase activity was determined (Figure 10). Four peaks were observed for the chiral separation of P4. Peaks 1 and 2 repres nted th *erythro*

diastereomers and 3 and 4 represented the *threo* diastereomers as determined by a sequential separation of the P4 mixture by reverse phase chromatography followed by the chiral separation. The enzyme activity was specifically inhibited by the fourth peak, the D-*threo* isomer (Figure 4A). This specificity for the D-*threo* enantiomer was consistent with the previous results observed in PDMP and PDMP homologues (2-4). The IC_{50} of D-*threo*-P4 was 0.5 mM for GlcCer synthase activity measured in the MDCK cell homogenates.

Effects of P4 and P4 derivatives with a single substituent of phenyl group on GlcCer synthase activity. The effect of each P4 isomer on GlcCer synthase activity was analyzed. The reaction was carried out in the absence or presence of 0.1, 1.0 or 10 μM P4 (Figure 4A) or p-methoxy-P4 (Figure 4B). As shown in Figure 4A, the enzyme activity was specifically inhibited by D-*threo* isomer. In Figure 4A, the symbols are denoted as follows: D-*threo* (o), D-*erythro* (□), L-*threo* and (•), L-*erythro* (▵). This specificity is consistent with previous results observed in PDMP and PDMP homologs. Inokuchi, J. et al., *J. Lipid. Res.* 28:565-571 (1987); Abe, A. et al., *J. Lipid. Res.* 36:611-621 (1995). The IC_{50} of D-*t*-P4 was 500 nM.

As set forth herein, the addition of a p-methoxy group to DL-*t*-P4 was found to enhance the effect of the inhibitor on the enzyme activity. Abe, A. et al., *J. Lipid. Res.* 36:611-621 (1995). As shown in Figure 4B, it was confirmed that the enzyme activity was potently inhibited by D-*threo*-p-methoxy-P4 whose IC_{50} was 200 nM. In Figure 4B, □ denotes a mixture of D-*erythro* and L-*threo* isomers contaminated with a small amount of the D-*threo* isomer. Chiral chromatography of the four p-methoxy-P4 enantiomers failed to completely resolve to baseline each enantiomer (Figure 10). A slight inhibition of the enzyme activity by p-methyoxy-P4 in a combined D-*erythro* and L-*threo* mixture (peaks 2 and 3, Figure 10) was observed; this was due to contamination of the D-*threo* isomer (peak 4, Figure 10) into these fractions.

A series of D-*t*-P4 derivatives containing a single substituent on the phenyl group were investigated. As shown in Table 8, the potency of the derivatives as inhibitors were inferior to that of D-*t*-P4 or p-methoxy-D-*t*-P4. In many drugs, the influence of an aromatic substituent on the biological activity has been known and predicted. Högberg, T. et al., *Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists.* Larsen, P.K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991). Generally IC_{50} is described as the following equation:

$$\log (1/IC_{50}) = a\,(\text{hydrophobic parameter } (\pi)) + b\,(\text{electronic parameter } (\sigma)) + c\,(\text{steric parameter}) + d\,(\text{other descriptor}) +$$

where a, b, c, d and e are the regression coefficients. Högberg, T. et al., *Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists.* Larsen, P.K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991).

The hydrophobic effect, π, is described by the equation $\pi = \log P_X - \log P_H$ where P_X is the partition coefficient of the substituted derivative and P_H is that of the parent compound, measured as the distribution between octanol and water.

The electronic substituent parameter, σ, was originally developed by Hammett (Hammett, L.P., In Physical Organic Chemistry, McGraw-Hill, New York (1940)) and is expressed as $\sigma = \log K_X - \log K_H$, where K_X and K_H are the ionization constants for a para or meta substituted derivative and benzoic acid respectively. Positive σ values represent electron withdrawing properties and negative σ values represent electron donating properties.

The potency of D-*threo*-P4 and P4 derivatives as an inhibitor is mainly dependent upon two factors, hydrophobic and electronic properties, of a substituent of phenyl group (Table 8). Surprisingly, a linear relationship was observed between log (IC_{50}) and $\pi + \sigma$ (Figure 5). These findings suggest that the more negative the value of $\pi + \sigma$, the more potent is D-*threo*-P4 derivatives made as GlcCer synthase inhibitor.

The data in Table 8 indicate that the potency of D-*t*-P4 and P4 derivatives as an inhibitor is mainly dependent upon two properties, hydrophobic and electronic properties, of a substituent of the phenyl group. Surprisingly, a linear relationship was observed between log(IC_{50}) and $\pi + \sigma$ (Figure 5). These findings suggest that the more negative the value of $\pi + \sigma$, the more potent the D-*t*-P4 derivative as a GlcCer synthase inhibitor.

Tabl 8

D-*threo*-P4 derivative	$\sigma + \pi$*	IC_{50} (μM)**
p-methoxy	-0.29	0.2
P-4	0.00	0.5
m-methoxy-P4	0.10	0.6
p-methyl-P4	0.39	2.3
p-chloro-P4	0.94	7.2

*These values were estimated from the Table in Högberg, T. et al., *Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists*. Larsen, P.K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991), for methoxy, $\sigma_m = 0.12$, $\sigma_p = -0.27$, $\pi = -0.02$; hydro, $\sigma = 0$, $\pi = 0$; methyl, $\sigma_p = -0.17$, $\pi = 0.56$; chloro, $\sigma_p = 0.23$, $\pi = 0.71$.

**These values were derived from Figures 4A and 4B. For other compounds the same analytical approach as shown in Figures 4A and 4B was carried out to obtain the IC_{50}.

The p-hydroxy-substituted homologue was a significantly better GlcCer synthase inhibitor. The strong association between $\pi + \sigma$ and GlcCer synthase inhibition suggested that a still more potent inhibitor could be produced by increasing the electron donating and decreasing the lipophilic properties of the phenyl group substituent. A predictably negative $\pi + \sigma$ value would be observed for the p-hydroxy homologue. This compound was synthesized and the D-*threo* enantiomer isolated by chiral chromatography. An IC_{50} of 90 nM for GlcCer synthase inhibition was observed (Figure 11), suggesting that the p-hydroxy homologue was twice as active as the p-methoxy compound. Moreover, the linear relationship between the log (IC50) and $\pi + \sigma$ was preserved (open circle, Figure 4).

Effects of 3',4'-dioxy-D-threo-P4 derivatives on GlcCer synthase activity. The result in Figure 5 suggested that an electron donating and hydrophilic substituent of phenyl group makes the GlcCer synthase inhibitor potent. To attain further improvement of the inhibitor, another series of P4 derivatives with methylenedioxy, ethylenedioxy and trimethyldioxy substitutions on the phenyl group were designed (Figure 9).

As shown in Figure 6, the enzyme activity was markedly inhibited by D-*t*-3',4'-thylenedioxy-P4 whose IC_{50} was 100 nM. In Figure 6, □ denot s D-*t*-3',4'-methylenedioxy-P4, o denotes D-*t*-3',4'-ethylenedioxy-P4, ▵ denot s D-*t*-3',4'-

trimethylenedioxy-P4 and • denotes D-*t*-3',4'-dimethyoxy-P4. One the other hand, the IC_{50}s for D-*t*-3',4'-methylenedioxy-P4 and D-*t*-3',4'-trimethylenedioxy-P4 were about 500 and 600 nM, respectively. These results suggest that the potency of D-*t*-3',4'-ethylenedioxy-P4 is not only regulated by hydrophobic and electronic properties but also by other factors, most likely stearic properties, induced from the dioxy ring on the phenyl group.

Interestingly, D-*t*-3',4'-dimethoxy-P4 was inferior to these dioxy derivatives, even to D-*t*-P4 or m- or D-*t*-p-methoxy-P4, as an inhibitor (Figure 6). As the parameters, σ_m, σ_p and π, for methoxy substituent are 0.12, -0.27 and -0.02, respectively (Högberg, T. et al., *Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists.* Larsen, P.K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991)), the value of $\pi + \sigma$ of D-*t*-dimethoxy P4 is presumed to be negative. Therefore the dimethoxy-P4 is thought to deviate quite far from the correlation as observed in Figure 5. There may be a repulsion between two methoxy groups in the dimethoxy-P4 molecule that induces a stearic effect that was negligible in mono substituent D-*t*-P4 derivatives studied in Figure 5. GlcCer synthase is thought to possess a domain that interacts with D-*t*-PDMP and PDMP homologs and that modulates the enzyme activity. Inokuchi, J. et al., *J. Lipid. Res.* 28:565-571 (1987); Abe, A. et al., *Biochim. Biophys. Acta* 1299:331-341 (1996). The stearic effect generated by an additional methoxy group may affect the interaction between the enzyme and the inhibitor. As a result, the potency as an inhibitor is markedly changed.

Distinguishing between inhibition of GlcCer synthase and 1-O-acylceramide synthase inhibition. Prior studies on PDMP and related homologues revealed that both the *threo* and *erythro* diastereomers were capable of increasing cell ceramide and inhibiting cell growth in spite of the observation that only the D-*threo* enantiomers blocked GlcCer synthase. An alternative pathway for ceramide metabolism was subsequently identified, the acylation of ceramide at the 1-hydroxyl position, which was blocked by both *threo* and *erythro* diastereomers of PDMP. Th specificities of D-*threo*-P4, D-*threo*-3',4'-ethylenedioxy-P4, and D-*threo*-(4'-hydroxy)-P4 for GlcCer synthase were studied by assaying the transacylase. Although there was an ca. 100 fold difference in activity between D-*threo*-3',4'-ethylen dioxy-P4, D-*threo*-(4'-hydroxy)-P4, and D-*threo*-P4 (IC_{50} 0.1 mM versus 10 mM) in inhibiting GlcCer synthase, the D-*threo* enantiomers of all three compounds demonstrated comparable activity in blocking 1-O-acylceramide synthase (Figure 12).

In order to determine whether inhibition of 1-O-acylceramide synthase was the basis for inhibitor mediated ceramide accumulation, the ceramide and diradylglycerol levels of MDCK cells treated D-*threo*-P4, D-*threo*-3',4'-ethylenedioxy-P4, and D-*threo*-(4'-hydroxy)-P4 were measured (Table 9). MDCK cells (5×10^5) were seeded into a 10 cm dish and incubated for 24 h. Following the incubation, the cells were treated for 24 or 48 h with or without P4 or the phenyl substitute homologues. Both ceramide and diradylglycerol contents were determined by the method of Preis, J. et al., *J. Biol. Chem.* 261:8597-8600 (1986). GlcCer content was measured densitometrically by a video camera and use of NIH image 1.49. Significant increases in both ceramide and diradylglycerol occurred only in cells treated with inhibitor concentrations in excess of 1 mM. This was approximately 30-fold lower than the concentration required for inhibition of the 1-O-acylceramide synthase assayed in the cellular homogenates. This disparity in concentration effects most likely reflects the ability of the more potent homologues to accumulate within intact cells. Abe, A. et al., *Biochim. Biophys. Acta* 1299:331-341 (1996).

Table 9
GlcCer, ceramide and diradylglycerol content of MDCK cells treated with D-*threo*-P4, D-*threo*-3',4'-ethylenedioxy-P4, and D-*threo*-(4'-hydroxy)-P4

Condition	Ceramide (pmol/nmol phospholipid)	Diradylglycerol (pmol/nmol phospholipid)
Control		
24 h	4.53 ± 0.12	24.2 ± 2.36
48 h	6.68 ± 0.49	32.3 ± 3.11
D-*threo*-P4		
11.3 nM		
24 h	5.33 ± 0.41*	24.1 ± 1.66
48 h	5.68 ± 0.27*	29.6 ± 0.73
113 nM		
24 h	4.64 ± 0.38	26.6 ± 1.56
48 h	7.08 ± 0.29	33.0 ± 2.63
1130 nM		
24 h	5.10 ± 0.35	27.1 ± 0.67
48 h	9.74 ± 0.53*	38.8 ± 1.11

D-*threo*-4'-hydroxy-P4		
11.3 nM		
24 h	4.29 ± 0.71	30.9 ± 2.01*
48 h	6.70 ± 0.29	38.4 ± 1.44*
113 nM		
24 h	5.09 ± 0.95	31.5 ± 3.84*
48 h	7.47 ± 0.29	41.5 ± 0.66*
1130 nM		
24 h	7.38 ± 0.13	38.5 ± 3.84*
48 h	13.4 ± 1.03*	47.2 ± 2.51*
D-*threo*-3',4'-ethylenedioxy-P4		
11.3 nM		
24 h	5.24	22.0
	5.04	24.7
113 nM		
24 h	5.21	32.5
	5.21	41.6
1130 nM		
24 h	9.64	32.5
	13.0	41.6

*Denotes $p < 0.05$ by the Student t test. For the D-*threo*-(ethylenedioxy)-P4 only two determinations were made.

Effects of D-threo-P4, D-threo-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth. To confirm the cellular specificity of D-*threo*-3',4'-ethylenedioxy-P4 and D-*threo*-(4'-hydroxy)-P4 as compared to D-*threo*-P4, MDCK cells were treated with different concentrations of the inhibitors. The total protein amount in each sample was determined by the BCA method. In GlcCer analysis, lipid samples and standard lipids were applied to the same HPTLC plate pre-treated with borate and developed in a solvent consisting of C/M/W (63/24/4). The level of GlcCer was estimated from a standard curve obtain d using a computerized imag scanner. The values were normalized on the basis of th phospholipid content. The results are shown in Figure 7, wherein ach bar is th average values from three dishes, with error bars corresponding to on standard

deviation. In the control, the total protein and GlcCer were 414 ± 47.4 μg/dish and 24.3 ± 1.97 ng/nmol phosphate, respectively.

Approximately 66 and 78% of the GlcCer was lost from the cells treated by 11.3 nM D-*threo*-4'-hydroxy-P4 and D-*threo*-3',4'-ethylenedioxy-P4 respectively (Figures 7, 14 and 15). By contrast, only 27 percent depletion of GlcCer occurred in cells exposed to D-*threo*-P4 (Figure 13). A low level of GlcCer persisted in the cells treated with 113 or 1130 nM of either compound. This may be due to the contribution, by degradation, of more highly glycosylated sphingolipids or the existence of another GlcCer synthase that is insensitive to the inhibitor.

On the other hand, there was little difference in the total protein content between untreated and treated cells with 11.3 or 113 nM nM D-*threo*-4'-hydroxy-P4 and D-*threo*-3',4'-ethylenedioxy-P4 (Figures 14 and 15). A significant decrease in total protein was observed in the cells treated with 1130 nM of either P4 homologue. In addition, the level of ceramide in the cells treated with 1130 nM D-*threo*-3',4'-ethylenedioxy-P4 and D-*threo*-(4'-hydroxy)-P4 was two times higher than that measured in the untreated cells (Table 9). There was no change in ceramide or diradylglycerol levels in cells treated with 11.3 nM or 113 nM concentrations of either compound. Similar patterns for GlcCer levels and protein content were observed at 48 h incubations.

The phospholipid content was unaffected at the lower concentrations of either D-*threo*-3',4'-ethylenedioxy-P4 or D-*threo*-(4'-hydroxy)-P4. The ratios of cell protein to cellular phospholipid phosphate (mg protein/nmol phosphate) were 4.94 ± 0.30, 5.05 ± 0.21, 4.84 ± 0.90, and 3.97 ± 0.29 for 0, 11.3, 113, and 1130 nM D-*threo*-3',4'-ethylenedioxy-P4 respectively, and 4.52 ± 0.39, 4.35 ± 0.10, and 3.68 ± 0.99 for 11.3, 113, and 1130 nM D-threo-4'-hydroxy-P4 suggesting that the changes in GlcCer content were truly related to inhibition of GlcCer synthase activity. These results strongly indicate that the inhibitors D-*threo*-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4, are able to potently and specifically inhibit GlcCer synthesis in intact cells at low nanomolar concentrations without any inhibition of cell growth.

SPECIFIC EXAMPLE 3

Compositions within the scope of invention include those comprising a compound of the pres nt invention in an effective amount to achieve an intended purpose. Determination of an effective amount and intended purpose is within the skill of the art. Preferred dosages are dependent for xample, on the s verity of the disease and the individual patient's response to the treatment.

As used herein, the term "pharmaceutically acceptable salts" is intend d to mean salts of the compounds of the present invention with pharmaceutically acceptable acids, *e.g.*, inorganic acids such as sulfuric, hydrochloric, phosphoric, etc. or organic acids such as acetic.

Pharmaceutically acceptable compositions of the present invention may also include suitable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which may be used pharmaceutically. Such preparations can be administered orally (*e.g.*, tablets, dragees and capsules), rectally (*e.g.*, suppositories), as well as administration by injection.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, *e.g.*, using the conventional mixing, granulating, dragee-making, dissolving or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as sugars, *e.g.*, lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, *e.g.*, tricalcium diphosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, *e.g.*, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose and/or polyvinylpyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, *e.g.*, silica, talc, stearic acid or salts thereof, such as magnesium stearat or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvent or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate, are used. Dyestuffs or pigments may be added to the tabl ts or dragee coatings, *e.g.*, for identification or in order to characterize different combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules may contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be used.

Possible pharmaceutical preparations which can be used rectally include, *e.g.*, suppositories, which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, *e.g.*, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols. It is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, *e.g.*, liquid triglycerides, polyethylene glycols or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, *e.g.*, water-soluble salts. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, *e.g.*, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension such as sodium carboxymethylcellulose, sorbitol and/or dextran. Optionally, the suspension may also contain stabilizers.

Alternatively, the active compounds of the present invention may be administered in the form of liposomes, pharmaceutical compositions wherein the active compound is contained either dispersed or variously present in corpuscles consisting of aqueous concentrate layers adherent to hydrophobic lipidic layer. The active compound may be present both in the aqueous layer and in the lipidic layer or in the non-homogeneous system generally known as a lipophilic suspension.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of th invention.

All publications cited herein are expressly incorporated by reference.

WE CLAIM:

1. A compound comprising D-*t*-3',4'- thylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptable salts thereof.

2. A compound comprising D-*t*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptable salts thereof.

3. A composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, and functional homologues, isomers and pharmaceutically acceptable salts thereof.

4. The composition of Claim 3, wherein the compound is D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

5. The composition of Claim 3, wherein the compound is a pharmaceutically acceptable salt of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

6. The composition of Claim 3, wherein the compound is D-*t*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

7. The composition of Claim 3, wherein the compound is a pharmaceutically acceptable salt of D-*t*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

8. A method for inhibiting the growth of cancer cells in a mammal, wherein said cancer cells are sensitive to the compounds below, comprising the step of administering to the mammal a therapeutically effective amount of a composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-ph nyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptabl salts thereof.

9. The method of Claim 8, where the growth of the cancer cells is inhibited by increasing ceramide levels in the cancer cells to a toxic level.

10. A method for treating a patient having sphingolipidosis by reducing glycosphingolipid synthesis comprising the step of administering to the patient a therapeutically effective amount of a composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptable salts thereof.

11. The method of Claim 10, wherein the patient is diagnosed as having Gaucher disease.

12. The method of Claim 10, wherein the patient is diagnosed as having Tay-Sachs disease.

13. The method of Claim 10, wherein the patient is diagnosed as having Fabry disease.

14. A method for treating a patient having a microbial or viral infection comprising the step of administering to the patient a therapeutically effective amount of a composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptable salts thereof.

15. A method for treating a patient having a drug resistant tumor sensitive to the compounds below, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptable salts thereof.

16. A method for reducing tumor angiogenesis in a patient, wherein said angiog nesis is sensitive to the compounds b low, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and functional homologues, isomers and pharmaceutically acceptable salts thereof.

17. A vaccination method comprising the steps of:

a) removing cancer cells sensitive to the compounds below, from a patient;

b) treating the cancer cells *in vitro* with an effective amount of a composition comprising a compound selected from the group consisting of D-*t*-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, D-*t*-4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol andfunctionalhomologues,isomers and pharmaceutically acceptable salts thereof; and

c) administering treated cells to the patient.

Figur 1

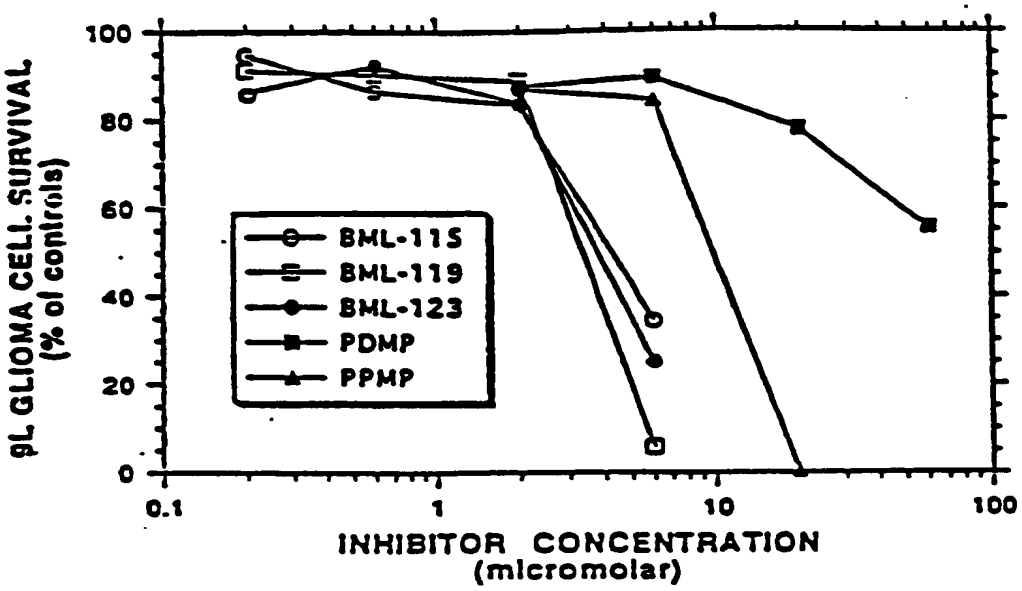


Figure 2

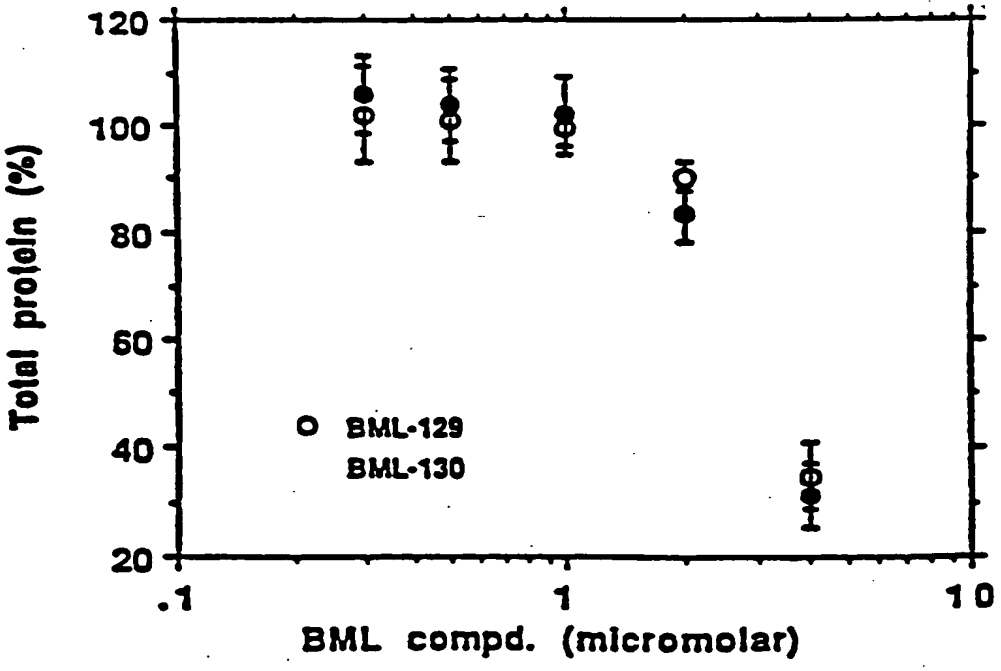
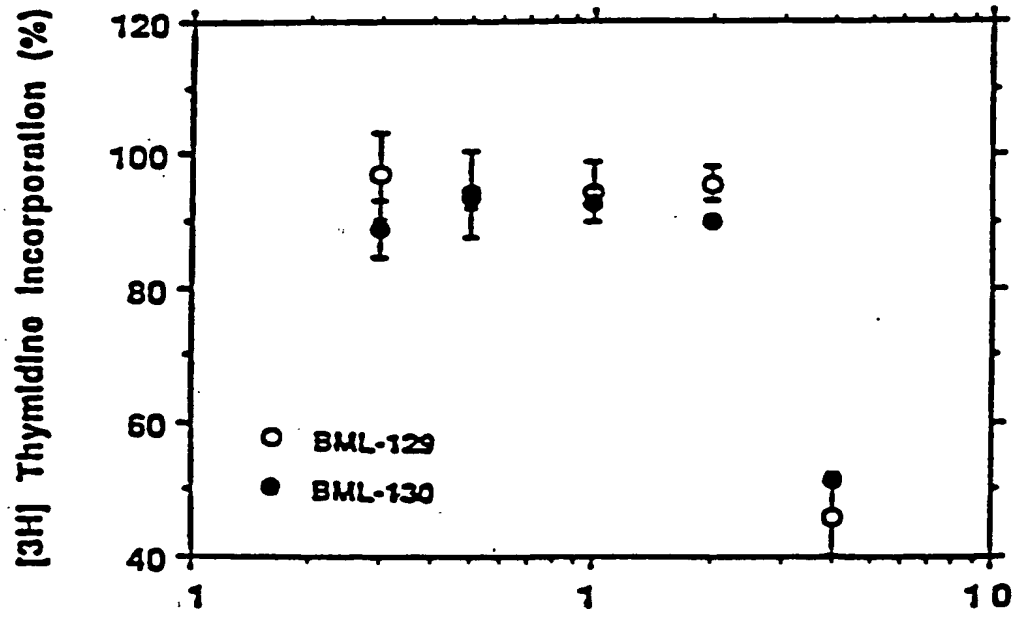


Figure 3


[3H] Thymidine Incorporation (%)
120
100
80
60
40
1
1
10
BML-129
BML-130

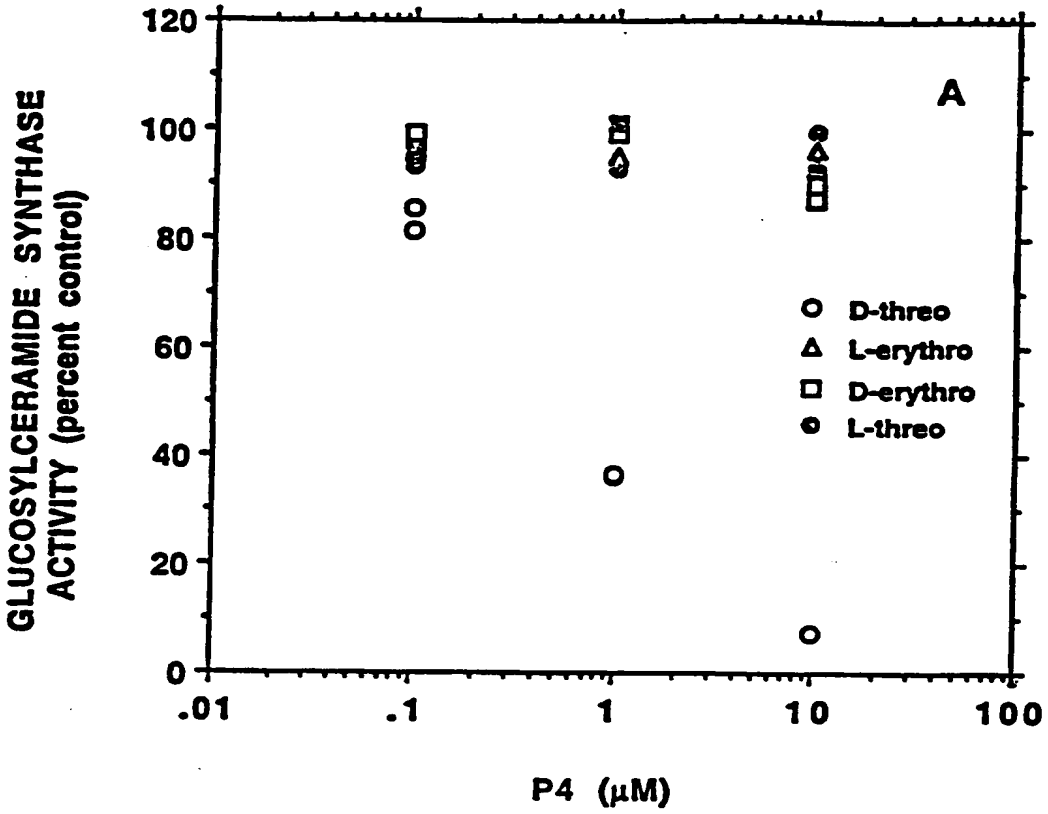

GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
120
100
80
60
40
20
0
.01
.1
1
10
100
P4 (μM)
A
D-threo
L-erythro
D-erythro
L-threo

Figure 4A

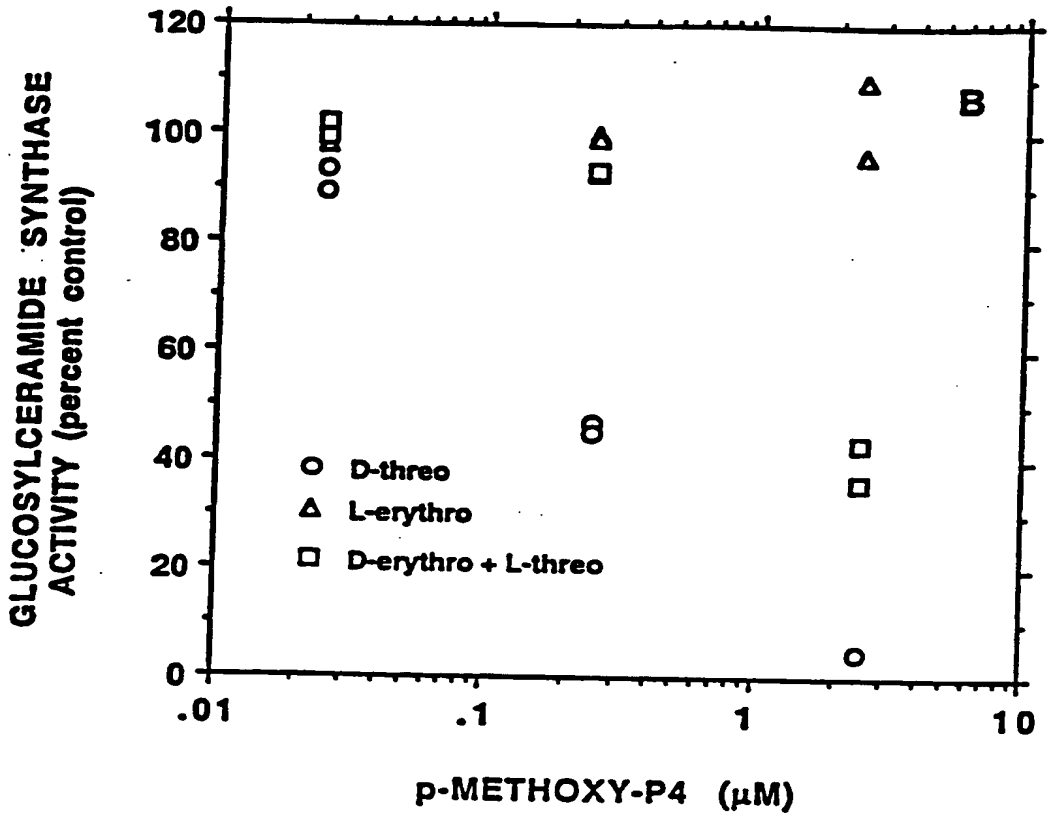

GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
120
100
80
60
40
20
0
.01
.1
1
10
p-METHOXY-P4 (μM)
D-threo
L-erythro
D-erythro + L-threo
B
8

Figure 4B

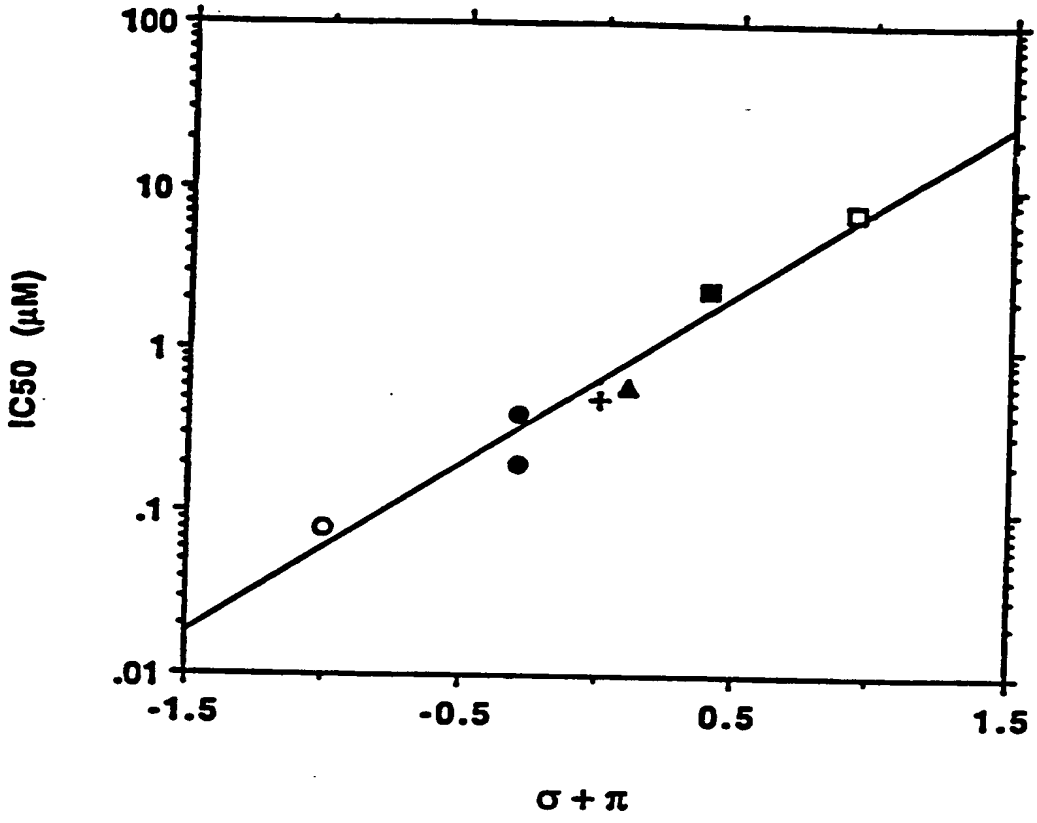


Figure 5

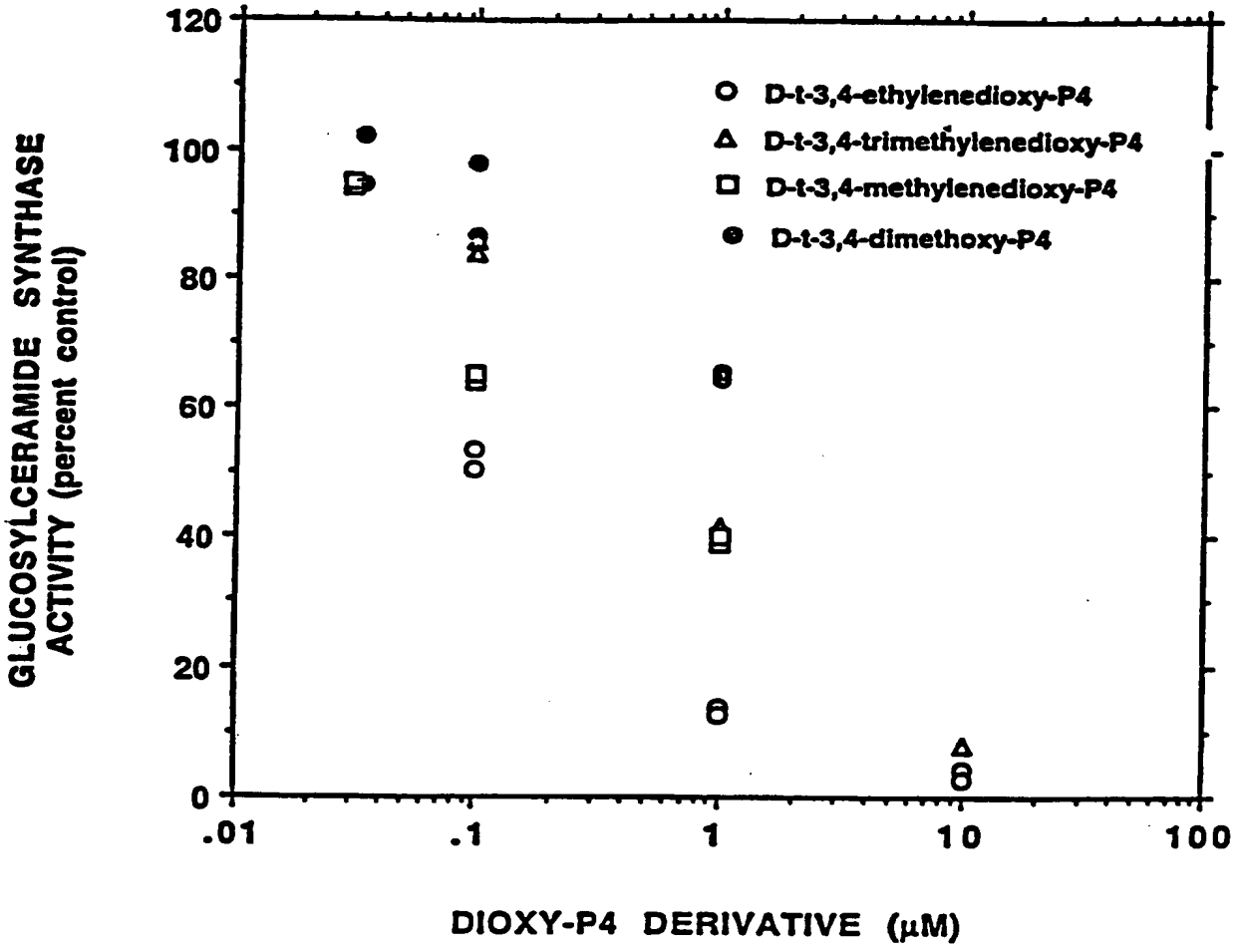

GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
120
100
80
60
40
20
0
.01
.1
1
10
100
DIOXY-P4 DERIVATIVE (μM)
D-t-3,4-ethylenedioxy-P4
D-t-3,4-trimethylenedioxy-P4
D-t-3,4-methylenedioxy-P4
D-t-3,4-dimethoxy-P4

Figure 6

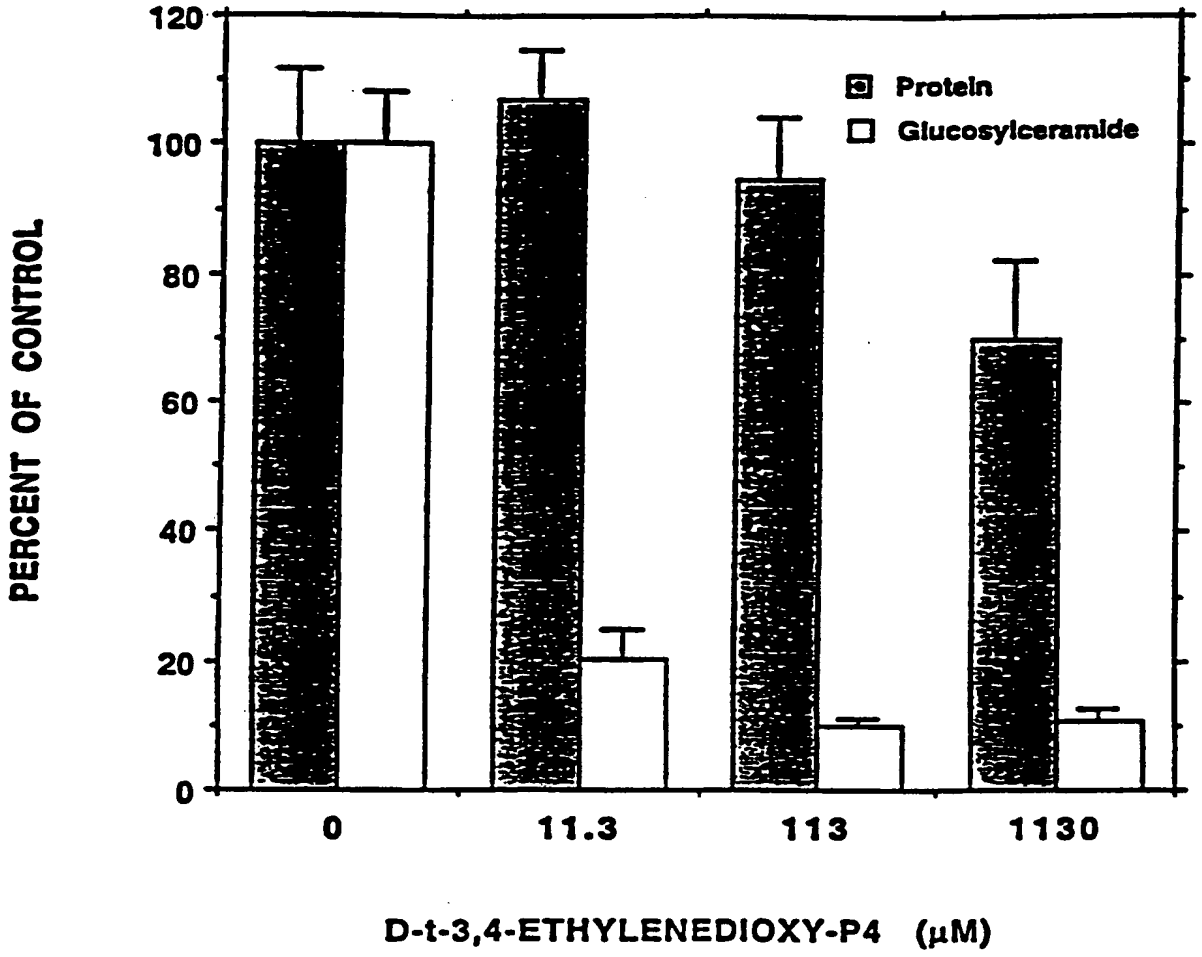

120
100
80
60
40
20
0
PERCENT OF CONTROL
Protein
Glucosylceramide
0
11.3
113
1130
D-t-3,4-ETHYLENEDIOXY-P4 (μM)

Figure 7

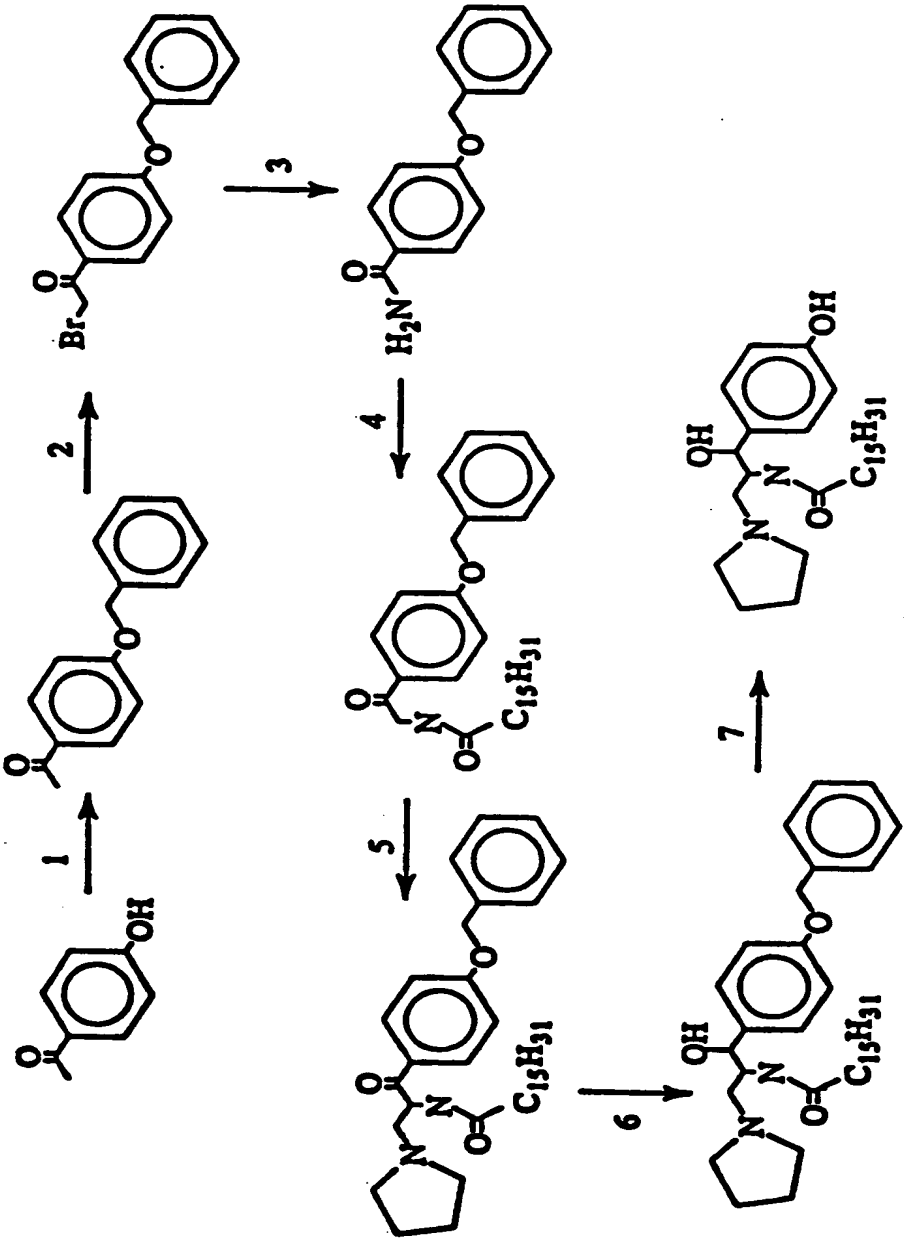
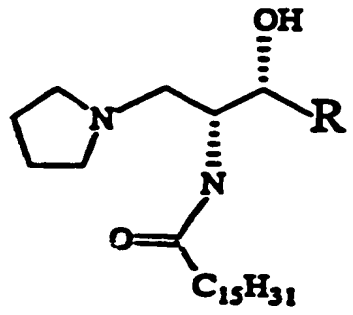
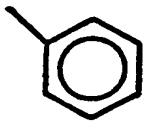


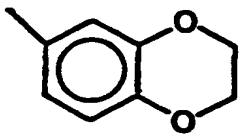
Figure 8



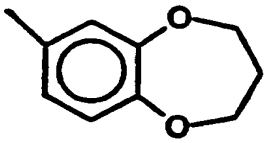
R=



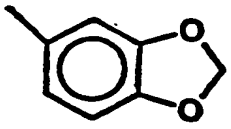
D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)



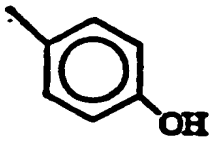
D-*threo*-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-1-(3',4'-trimethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-1-(3',4'-methylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)

Figure 9

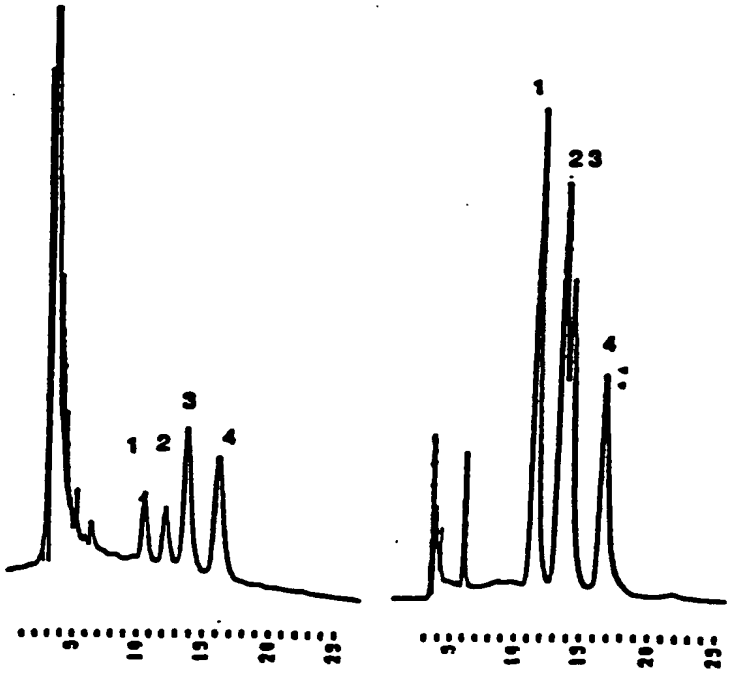


Figure 10

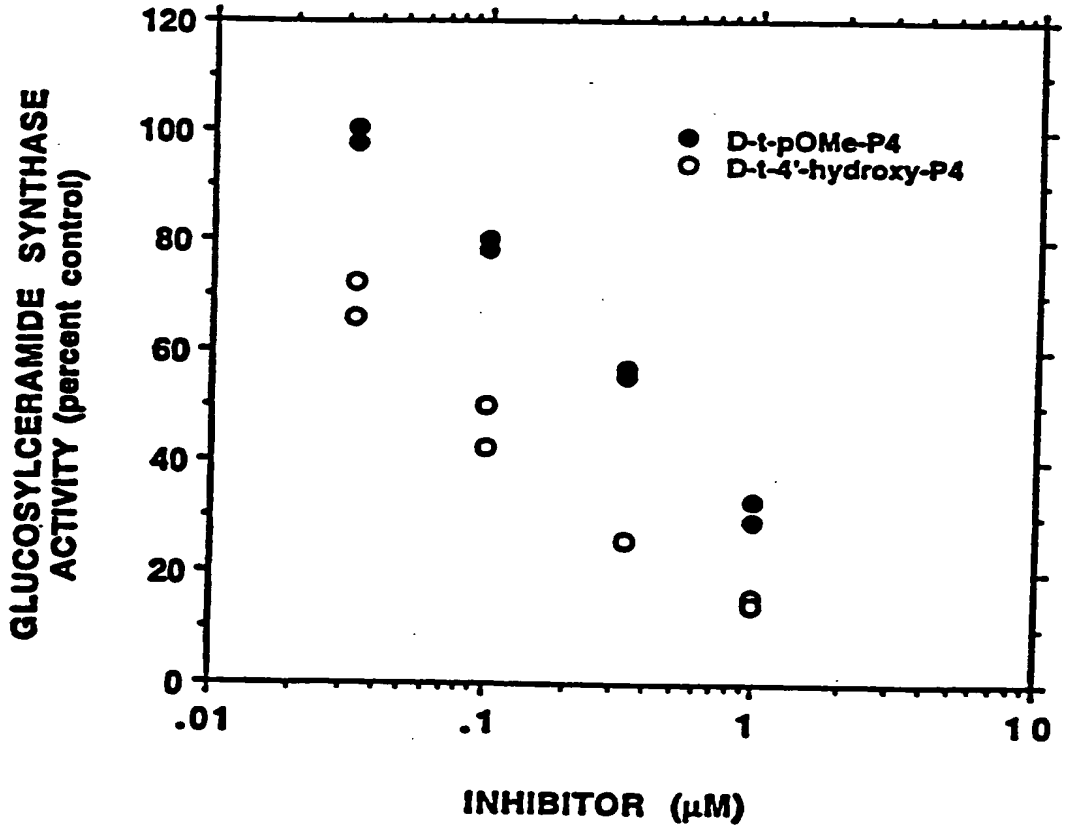


Figure 11

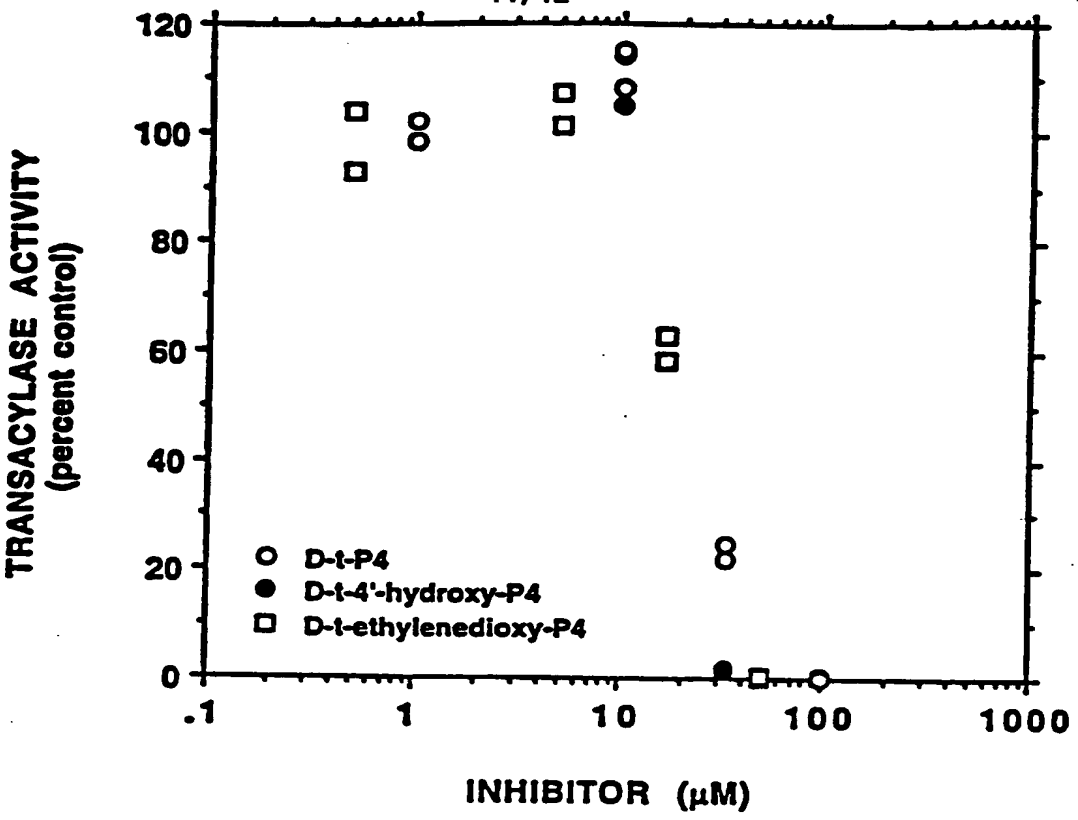


Figure 12

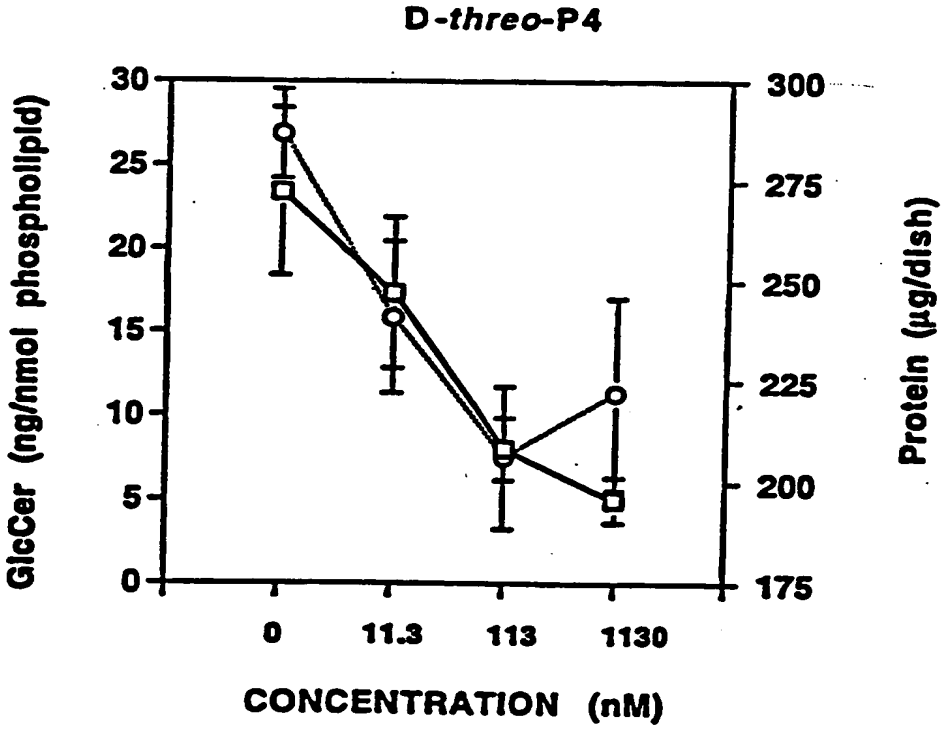


Figure 13

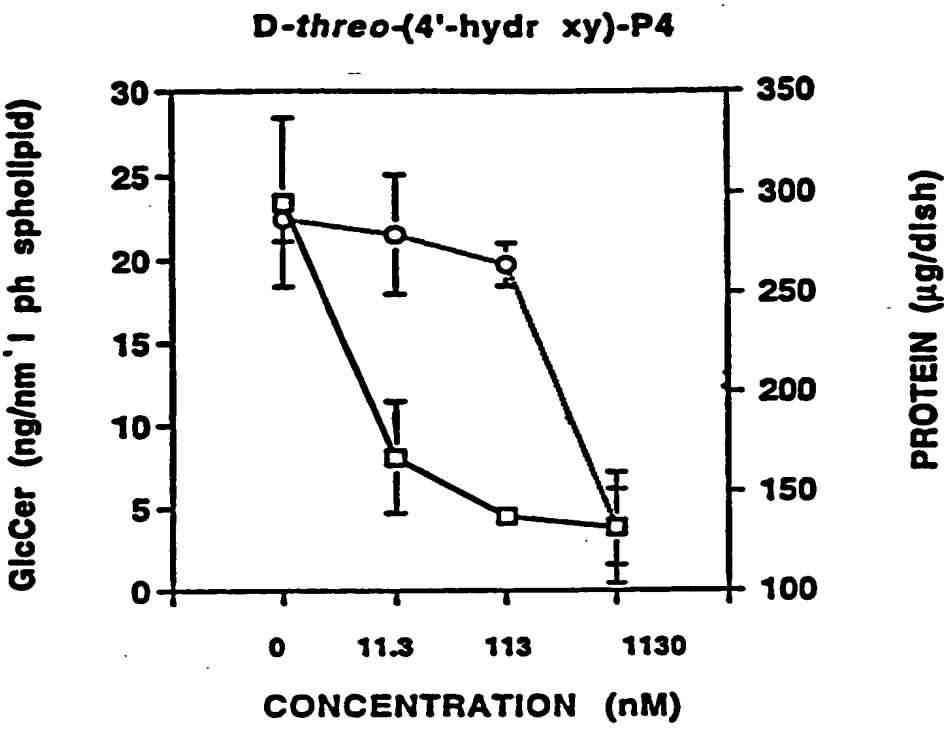


Figure 14

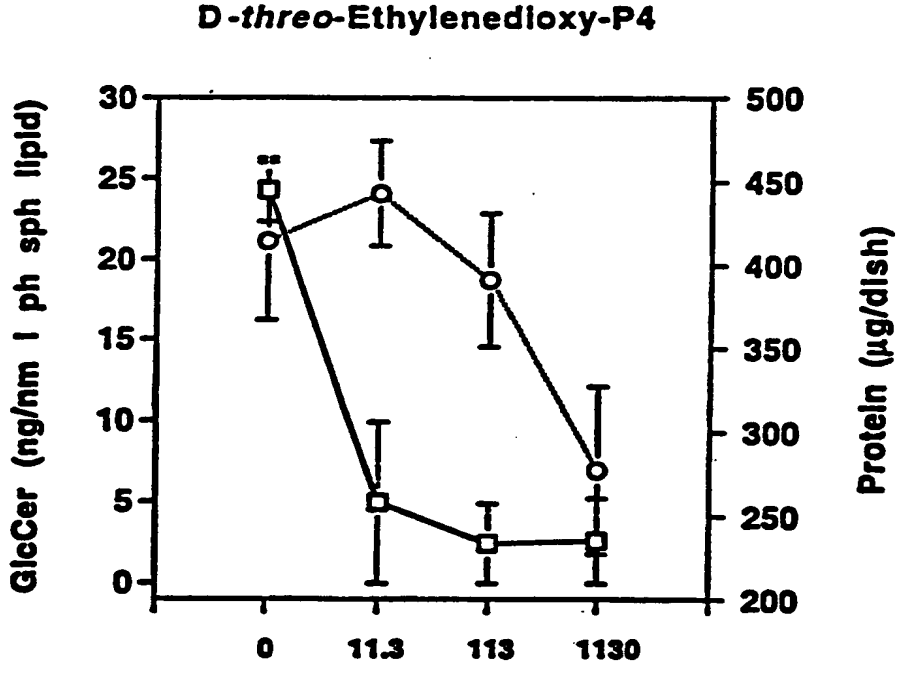


Figure 15

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 00/18935

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D319/18 C07D295/12 A61K31/40 A61K31/4025 A61P35/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BEILSTEIN Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE, LIHSUEH ET AL: "Improved inhibitors of glucosylceramide synthase" J. BIOL. CHEM. (1999), 274(21), 14662-14669 , XP002151667 figure 2	1-17
A	WO 97 10817 A (UNIV MICHIGAN) 27 March 1997 (1997-03-27) page 4, line 28 -page 5, line 15 & US 5 916 911 A (RADIN NORMAN S ET AL) 29 June 1999 (1999-06-29) cited in the application	1-17

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 2000	28/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Seitner, I

INTERNATIONAL SEARCH REPORT

information on patent family members

Intern[illegible]nal Application No
PCT/US 00/18935

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9710817 A	27-03-1997	US 5916911 A	29-06-1999
		US 5952370 A	14-09-1999
		US 5945442 A	31-08-1999